A NOVEL STEM LEYDIG CELL CULTURE SYSTEM AND ITS USE IN ASSESSING THE EFFECTS OF PHTHALATE EXPOSURE ON LEYDIG CELL DEVELOPMENT

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ABSTRACT

After eliminating Leydig cells in the adult rat testis with ethane dimethanesulfonate (EDS), a new population of testosterone-producing Leydig cells forms, arising from stem cells. Using isolated rat seminiferous tubules, we showed that stem cells associated with the tubule surfaces first proliferated and then differentiated in response to culture with LH. Specific tubule-secreted factors were shown able to enhance proliferation and differentiation. In particular, the Sertoli cell products FGF2 and PDGFBB increased the proliferation of the stem Leydig cell population during the first week of culture, and Desert hedgehog (DHH) was found to commit the stem cells to a differentiation pathway during weeks 2−3. With regard to the latter, 3βHSD+ cells formed on the surfaces of the tubules in response to DHH that produced testosterone (T), and continued to do so for many weeks.

These results suggested that stem cells residing on the surface of the tubules are influenced by endogenous factors coming from the tubules. We realized that if this was the case, the use of cultured tubules would not allow the effects of specific, added factors on stem cell proliferation and/or differentiation to be determined. We therefore sought to isolate stem cells from the tubules as well as the interstitial tissue. A heterogeneous population of cells, including stem Leydig cells, that when isolated from the tubules and cultured in vitro, were found to first proliferate in response to FGF2 and PDGFBB in the culture medium, as measured by EdU labeling, and then to differentiate in response to Desert hedgehog (DHH) to produce testosterone. Thus, the temporal separation observed when culturing whole tubules could be maintained when culturing isolated stem Leydig cells.

We used both cultured tubules and cells isolated from the tubules to assess the effects of the metabolite of diexylhexyl phthalate (DEHP), metabolite mono2-ethylhexyl) phthalate (MEHP) on the proliferation and on differentiation of the stem Leydig cells. Tubules were cultured with LH alone, LH + 0.2 μg/ml MEHP or LH + 1 μg/ml MEHP for up to 24 days. A dose-dependent decrease in testosterone production was seen in response to MEHP. With stem cells obtained from seminiferous tubules, in vitro culture with LH for 1 week resulted in cell proliferation. When the isolated cells were cultured with LH + 0.1 μM MEHP, greater numbers of EdU-labeled cells were apparent after 1 week compared to culture with LH alone. When 2 μM DHH was added after week 1, testosterone production was greater by cells that had been cultured with MEHP during week 1 than cells cultured with LH alone, perhaps reflecting the greater numbers of cells resulting from MEHP exposure. To examine phthalate effects on cell differentiation, the cells were incubated in LH (10 ng/ml) for week one, and then during week 2 with LH alone or LH plus MEHP of increasing dose, and finally with 2μM DHH. Phthalate exposure during the second week had inhibitory effects on T production compared to LH−alone controls, with the higher doses more inhibitory than lower doses. These studies indicate that: 1) the novel system developed for examining stem Leydig cell function should be useful in studying the effects of environmental toxicants on Leydig cell development and function; 2) as with cultured seminiferous tubules, isolated stem Leydig cells cultured in vitro can be induced to first proliferate (week 1) and subsequently differentiate (weeks 2-3); that is, the temporal separation observed when culturing whole tubules is maintained when culturing isolated stem Leydig cells; 3) the cells can be induced to proliferate by culture with PDGFBB and FGF2; 4) cells can be induced to differentiate by culture with DHH; 5) the isolated cells will continue to produce

testosterone for long periods of time; 6) the isolated cells can be used to examine factors that influence their proliferation and differentiation in the absence of influence from the seminiferous tubules; and 7) this system provides a way to assess the effects of exposure to environmental toxicants on Leydig cell differentiation without the influence of other cells or organs.

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LITERATURE REVIEW

Leydig Cell Structure and Function

Leydig cells are the cells in the interstitial region of the testes responsible for the production of testosterone [Chen et al, 2009; Habert et al, 2001]. It has been determined that there are two distinct populations of Leydig cells in the testis throughout the life of a male [Habert et al, 2001; Kerr and Knell, 1988; Teerds et al, 2007]. First are the fetal Leydig cells. These cells are responsible for producing masculinizing doses of testosterone necessary for male development [Habert et al, 2001; O'shaughnessy et al, 2006]. For instance, testosterone produced by fetal Leydig cells is essential for the differentiation of the male urogenital system and for scrotal descent of the testis [Chen et al, 2009; Habert et al, 2001; O'shaughnessy et al, 2006; Zhang et al, 2008]. There is a peak in fetal androgen production just prior to birth to ensure proper masculinization of the fetus [O'shaughnessy et al, 2006]. The majority of this cell population regresses after the initial masculinization is complete, but some fetal Leydig cells are believed to persist into adult life [Habert et al, 2001; Kerr and Knell, 1988]. There are three main morphological features that distinguish fetal Leydig cells from other cell types of the fetal testis and from the adult Leydig cells. They have an irregularly-shaped oval nucleus with a nucleolus, there is a visible rim of cytoplasm, and they have an abundance of cytoplasmic lipid inclusions for steroid biosynthesis [Kerr and Knell, 1988]. The fetal Leydig cells also form clusters and bundles [Kerr and Knell, 1988]. These cells express the luteinizing hormone (LH) receptor and respond to LH from the anterior pituitary gland, but they do not require LH stimulation to produce testosterone [Zhang et al, 2008]. This is just one of many factors that sets fetal Leydig cells apart from their successors, adult Leydig cells.

In humans, adult Leydig cells form from puberty to adulthood and are the primary source of testosterone production in males [Chen et al, 2009; Zhang et al, 2008]. The adult Leydig cells are significantly larger than the fetal Leydig cells and are not bundled together [Habert et al, 2001, Kerr and Knell, 1988]. They contain few cytoplasmic lipid inclusions, but rather have a greater abundance of smooth endoplasmic reticulum and mitochondria [Kerr and Knell, 1988]. The testosterone produced by the adult Leydig cells provides the androgens necessary for spermatogenesis and many other male reproductive functions [Chen et al, 2009; Habert et al, 2001; Kerr and Knell, 1988; Zhang et al, 2008]. Unlike the fetal Leydig cells, adult Leydig cells are absolutely dependent on LH from the anterior pituitary gland to synthesize testosterone [Habert et al, 2001; O'shaughnessy et al, 2006; Teerds et al, 2007].

Testosterone production in the male is regulated by the hypothalamic-pituitary-gonadal axis. Gonadotropin-releasing hormone (GnRH) is produced by the hypothalamus, and acts on the anterior pituitary to stimulate the release of gonadotropins LH and follicle stimulating hormone (FSH) into circulation throughout the body [Ivell et al, 2014]. These peptide hormones bind specific cell-surface receptors in the testes to carry out their effects. FSH in males plays a crucial role in initiating spermatogenesis. LH is critically involved in testosterone production by ALCs. Specifically, LH binds the LH receptor (LHR) on Leydig cells to stimulate testosterone synthesis. LH binding the LHR and then coupling to G-protein-coupled receptors (GPCRs) stimulates the production of adenylate cyclase, which results in cAMP production. cAMP then stimulates steroid hormone biosynthesis through a cholesterol-mediated pathway [Ivell et al, 2014]. When there is enough testosterone sensed in the body, its presence negatively feeds back and temporarily inhibits the hypothalamus from releasing more GnRH, so no LH/FSH is

released, and testosterone is no longer made [Chimento et al, 2014]. When testosterone levels begin to get too low, the hypothalamus is no longer inhibited by testosterone, so it releases GnRH and the cycle of testosterone production resumes [Chimento et al 2014]. This negative feedback cycle of pulses of GnRH stimulating pluses of LH stimulating pulses of testosterone is responsible for the maintenance of testosterone levels in the body.

The building block of all steroid hormones is cholesterol, and its conversion into steroid hormones is tightly regulated by various enzymes in the mitochondria and smooth endoplasmic reticulum [Habert et al, 2001; Martinez-Arguelles et al, 2013]. The rate-limiting step of steroidogenesis is the import of cholesterol (either from the extracellular space or made de novo within the cell) into the mitochondria [Jefcoate, 2006; Martinex-Arguelles et al, 2014; Rone et al, 2009]. This key step is facilitated by steroidogenic acute regulatory protein (StAR) and translocator protein (TSPO) after the cell has responded to LH [Habert et al, 2001; Martinez-Arguelles et al, 2013]. Once in the inner mitochondrial membrane, cholesterol is cleaved into pregnenolone by P450scc (CYP11A1). From here, pregnenolone enters the smooth ER where, through a series of enzymatic reactions, testosterone is synthesized [Habert et al, 2001; Martinez-Arguelles et al, 2013] (Fig. 1).

Stem Cells

Stem cells are defined by their unique ability to both self-renew and differentiate into various cell types in the body [Zhang et al, 2008]. There are different classes of stem cells, based on their differentiation capacity. Stem cells are classified as totipotent, pluripotent, multipotent, or unipotent [Zhang et al, 2008].

Stem cells first differentiate into progenitor cells, which have the ability to commit to and develop into specific cell types [Kumar et al, 2010]. The potency of a stem cell will dictate how many cell types that stem cell can differentiate into. Totipotent stem cells have the ability to differentiate into all the embryonic and extraembryonic cell types of the body [Kumar et al, 2010]. Pluripotent stem cells are the next class of stem cells. They have the ability to differentiate into any cell derived from the mesoderm, endoderm and ectoderm [Kumar et al, 2010]. Multipotent stem cells can differentiate into cell types within a specific family of cells [Kumar et al, 2010]. Lastly, unipotent stem cells can differentiate into only one specific cell type, while continuing to self-renew to maintain the cell population [Kumar et al, 2010].

Adult stem Leydig cells have been reported to localize to both the peritubular and perivascular regions of the interstitial space of the postnatal testis [Zhang et al, 2008]. In rodents, there have been two generations of Leydig cells identified, as mentioned above—fetal Leydig cells and adult Leydig cells [Zhang et al, 2008]. Fetal Leydig cells are present during gestation, and differentiate from a fetal stem Leydig cell population [Zhang et al, 2008]. This cell population is essential for masculinization of the fetus. By embryonic day 12 in rats, the fetal Leydig cells are committed and have begun differentiating leading to testosterone biosynthesis [Zhang et al, 2008]. After birth, however, fetal Leydig cells regress and contribute negligible amounts of androgens to the male [Zhang et al, 2008].

Adult Leydig cells form in three phases from adult Leydig stem cells that are present in the testis at birth [Ge et al, 2006; Zhang et al, 2008]. The formation of these cells requires cell proliferation, morphological differentiation, and the ability of the cells to produce testosterone [Benton et al, 1995]. The progression of these cells through the three developmental stages is

monitored by the presence (or absence) of stem cell specific markers, and/or Leydig cell-specific markers. For example, the expression of Leydig-cell specific marker, 3βHSD is used to demonstrate cells that have committed to the Leydig cell lineage [Zhang et al, 2008]. The stem Leydig cells express genes for platelet derived growth factor receptor-α (PDGFR-α), which is specific for Leydig precursor cells [Ge et al, 2006; Odeh et al, 2014]. They also are positive for stem cell factors such as leukemia-inhibitory factor (LIF), the LIF-receptor and c-kit, and for GATA-binding protein 4, which is a Leydig cell lineage marker. They do not express 3βHSD or the LHR, demonstrating their lack of differentiation [Ge et al, 2006; Odeh et al, 2014; Zhang et al, 2008]. The first phase that these stem cells transition into is the progenitor Leydig cells, which divide a rapid but finite number of times [Zhang et al, 2008]. These cells form after proliferation of the stem cells. They express 3βHSD and commit to the progenitor phase by the second week of postnatal life in rats [Benton et al, 1995; Ge et al, 2006; Zhang et al, 2008]. These cells also express mRNA and proteins that are essential for steroidogenic function including P450scc, P450c17, and the LHR [Zhang et al, 2008]. These cells must be stimulated with LH to produce low levels of testosterone. While they do not produce much testosterone, they have high rates of testosterone metabolism [Chen et. al, 2009; Ge et al, 2006].

Following the progenitor Leydig cell stage is the immature Leydig cell stage (ILC). In rat testes, these cells are usually seen between PND28-56 [Zhang et al, 2008] A defining feature of cells in this stage is that they contain abundant smooth endoplasmic reticulum and have increased amounts of lipid droplets, to allow for the production of steroid hormones [Zhang et al, 2008]. These morphologic changes closely resemble the structure of an adult Leydig cell [Benton

et al, 1995]. The primary androgen produced at this stage is 5α -androstanediol [Benton et al, 1995; Zhang et al, 2008].

Finally, by day 56 in rats, adult Leydig cells are seen [Benton et al, 1995; Zhang et al, 2008]. Morphologically, these are large cells with copious amounts of smooth ER, few lipid droplets, a large nucleolus and high levels of steroidogenic enzymes to facilitate the production of testosterone [Benton et al, 1995; Zhang et al, 2008]. This cell population appears at puberty and the testosterone produced enables spermatogenesis to occur and aids in maintaining male reproductive health and function [Zhang et al, 2008]. Adult Leydig cells are the chief cell population in a mature testis. In the rat, there are approximately 25 million Leydig cells [Benton et al, 1995; Zhang et al, 2008]. The adult Leydig cells have a low rate of turnover. However, if they are destroyed they do have regenerative capabilities due to the persistent stem cell population present in the adult testis [Benton et al, 1995; Teerds et al, 1999]. Markers specific for adult Leydig cells include 3βHSD, insulin-like growth factor 3, and LHR expression, as well as other genes necessary for steroid biosynthesis such as P450scc and P450c17 [Stanley et al, 2011].

While there are countless populations of stem cells in the body, each population is established in a specific niche. The niche is defined by both its anatomic location in the body and its function [Scadden, 2006]. Anatomically, the niche provides a habitat or architecture for the stem cells to reside, while functionally, its role is to regulate the stem cell population and facilitate differentiation and tissue development or regeneration whenever necessary [Scadden, 2006]. Often, the niche is comprised of a mix of cell types and extracellular-matrix proteins that comprise a three-dimensional space [Scadden, 2006]. The niche influences stem cell decisions

and fate by facilitating the secretion of various paracrine factors [Scadden, 2006]. For Leydig cells, the seminiferous tubules have recently been identified as a potential Leydig stem cell niche [Li et al, 2016; Stanley et al, 2011]. Adult Leydig cells in the interstitial space communicate closely with neighboring seminiferous tubules. They provide the tubules with the testosterone necessary to facilitate spermatogenesis while the tubules secrete various factors that influence the fate of the stem cells—either facilitating their proliferation or differentiation. In terms of regulating stem Leydig cell development, there are certain niche-specific and Sertoli cell secreted factors that influence their proliferation and differentiation in a temporally distinct fashion [Li et al, 2016].

Phthalates

Phthalates—synthetic diesters of phthalic acid—are a family of plasticizing chemicals that are used in various medical devices and consumer products [Buckley et al, 2012; Kavlock et al, 2006; Schettler, 2006]. Products they are used in include children's toys, furnishing, clothing, detergents, medical devices, food packaging/storage containers, cars, and cleaning materials [Buckley et al, 2012; Kavlock et al, 2006; Kilcoyne et al, 2014]. They are used both to provide rigidity to polymeric materials, and to act as solvents to provide lubrication/flexibility [Schettler, 2006]. The two most abundant phthalates used are di-n-butyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP) [Zhang et al, 2008]. For example, DEHP is added to many medical devices made from polyvinyl chloride to increase their flexibility [Akingbemi et al, 2001; Kavlock et al, 2006; Latini et al, 2004; Schettler, 2006]. Because they are used in so many facets—there are more than three million metric tons of phthalates produced around the world

each year—phthalate exposure is extremely widespread in the developed world [Buckley et al, 2012; Schettler, 2006].

The concern surrounding phthalate exposure is that phthalates are endocrine disrupting compounds with the potential to impair human reproductive and developmental health [Akingbemi et al, 2001]. The monoester metabolites of phthalates such as MEHP and MBP, the metabolites of DEHP and DBP, respectively, are what cause reproductive toxicity, in part through their actions as anti-androgens. [Borch et al, 2006; David, 2006; Huand et al, 2012]. However, while phthalates are classified as anti-androgens, they do not act through the androgen receptor [Zhang et al, 2008]. Important windows of sensitivity to phthalate exposure occur in utero and during lactation [Zhang et al, 2008]. Phthalates have been shown to disrupt Leydig cell structure and function in males, thus leading to abnormal testosterone production, and resulting developmental abnormalities such as hypospadias, cryptorchidism and impaired spermatogenesis which comprise Testicular Dysgenesis Syndrome (TDS), infertility, and possibly even cancer [Akingbemi et al, 2001; Borch et al, 2006; David, 2006; Huang et al, 2012; van den Driesche et all, 2014].

Due to their ubiquitous use, there are many routes through which humans can be exposed to phthalates. For example, the chemical can be ingested from food stored in containers made with phthalates because phthalates are able to leach out of the plastic in which they are used [Akingbemi et al, 2001; David, 2006; Fischer et al, 2013; Kavlock et al, 2006; Schettler, 2006]. Additionally, phthalates can be inhaled, absorbed through the skin, passed through bodily fluids, or encountered through ambient environmental contamination [Schettler, 2006]. For the general adult human population, ambient environmental exposures to phthalates are 3-30ug/kg bw/day

[Schettler, 2006]. Infants and toddlers, however, may have exposures several fold higher, especially those undergoing therapeutic medical interventions [Schettler, 2006].

Evidence from human and animal studies supports the contention that fetal programming can influence adult testosterone production. It has been shown that programing can be affected and, with respect to Leydig cells, manifest by causing decreased levels of testosterone production and possibly reproductive disorders in the adult male. [Drake et al, 2009; Eisenberg et al, 2012; Kilcoyne et al, 2014; Vanbillemont et al, 2010]. We have hypothesized that early exposure to phthalates might affect the Leydig stem cells present in the fetus and in infant males, resulting in changes in the proliferation and/or differentiation of these cells and thus in altered steroidogenic function of the adult Leydig cells.

Experiments have shown that phthalates, specifically DEHP and DBP, act in a time, dose, and age-dependent manner, and the effects of exposure are therefore determined by the stage of development of the fetus or adult [Latini et al, 2004]. Fetal and infant exposures are of greatest concern because DEHP, DBP, and other phthalates have the potential to have the most harmful effects on these populations. The most common sources of infant exposure to phthalates are breast milk, infant formula, baby food, and air containing DEHP coming in contact with the skin and/or mouth [Latini et l, 2004]. Congenital cryptorchidism is one of the reproductive disorders that falls under the umbrella of TDS, and has been shown to be associated with in utero and infant phthalate exposures. For example, a Danish study found a correlation between phthalates in breast milk and increased incidence of cryptorchidism [Krysiak-Baltyn et al, 2012]. An additional study looked at the relationship between phthalates in maternal breast milk and altered endogenous reproductive hormone secretion in their male infants. This study demonstrated that

the monoester metabolites of phthalates affected Leydig cell function [Main et al, 2006]. Specifically, there was a dose-dependent, negative correlation between metabolite concentration in breast milk and free testosterone production in infant males [Main et al, 2006].

Exposure is dramatically increased for infants who are in the neonatal intensive care unit because DEHP is frequently used to make the medical devices that are used on these babies for prolonged periods of time [Fischer et al, 2013; Latini et al, 2004]. Additionally, these infants often have immature metabolic systems, specifically reduced cytochrome p450 function, and therefore are at risk for extended exposure to phthalates and their metabolites [Fischer et al, 2013]. Neonatal exposure occurs most often through the use of different medical tubes manufactured with phthalates such as nasogastric tubes, enteral feeding tubes, umbilical catheters, oxygenation tubing, and endotracheal tubing [Fischer et al, 2014; Weuve et al, 2006]. Additionally, due to the leeching nature of phthalates, bags used to distribute blood and other fluids and gloves used by physicians can become contaminated with DEHP [Fischer et al, 2014; Weuve et al, 2006]. The main concern for exposed neonates is altered male reproductive function [David, 2006; Fischer et al, 2014]. Studies performed in rats showed that neonatal exposure results in testicular damage, seminal duct atrophy, germ cell degradation and hypo-fertility [Fischer et al, 2014]. The mechanisms for these adverse effects, however, are still under investigation, and will be addressed later in this review.

In addition to infant exposures are the issues of prenatal/in utero exposures, and how exposure to phthalates during gestation can cause lifelong adverse effects. When a pregnant mother is exposed to phthalates, studies show that the chemical penetrates the placenta and thus exposes the child [Huang et al, 2012]. It is during these critical and sensitive windows of

development of the reproductive tract during pregnancy that endocrine disrupting compounds, such as phthalates, act to alter normal developmental processes and have a toxic effect. In the case of phthalates in utero exposure leads to irregular sexual differentiation and testosterone production, and subfertility later in life, implying that these in utero exposures may be affecting a cell population that persists throughout the adult male life [Bornehag et al, 2015; Huang et al, 2012].

While there is correlational evidence between endocrine disruptor exposure and increased incidences of testicular cancer, TDS, and reduced semen quality, laboratory in vitro and in vivo studies are necessary to identify the mechanisms through which phthalates act to cause adverse reproductive health outcomes in males. Rodents are used as a model organism for these studies because phthalates are a known male reproductive toxicant in rats, and a major target of toxicity is the developing testis [Mylchreest et al, 1990; Mylchreest et al, 1999; Mylchreest et al, 2000; Shultz et al, 2001]. Various studies have been performed to examine the toxicity of phthalates at different doses and at different stages of in utero and postnatal development. Major issues are identifying the mechanism behind which these in utero exposures impair Leydig cell development and function, and how this ultimately leads to the development of TDS, infertility, and other reproductive disorders in males at different stages of life.

Stem Leydig Cell Identification and Classification

Leydig cells are now known to differentiate from precursor cells, previously referred to as the progenitor Leydig cell (PLC) population. The PLCs are 3βHSD+ and LHR+ [Ge et al, 2006]. A series of experiments by Ge et al. provided evidence that the PLCs derive from an

undifferentiated SLC population that is present in the neonatal testis. SLCs are 3βHSD and LHR-negative, but PDGFRα-positive [Ge et al, 2006].

Initial in situ experiments were performed to identify the putative SLC population in the neonatal testis. On postnatal day 7 (PND7), testis sections revealed spindle-shaped cells were 3βHSD-negative but BrdU positive, demonstrating that the cells they identified were actively dividing [Ge et al, 2006]. These cells were localized to the peritubular layer of the testis sections [Ge et al, 2006]. However, by PND14, the spindle-shaped cells in the peritubular layer were now 3βHSD-positive, denoting their transition from putative SLCs to PLCs [Ge et al, 2006]. Subsets of cells were also BrdU positive, confirming that they were derived from the originally 3βHSD-negative putative SLC population [Ge et al, 2006].

The next experiment sought to characterize the putative SLCs from the neonatal testis and assessed their ability to both proliferate and differentiate, which would give them the necessary characteristics to be considered a stem cell. Putative SLCs were isolated on PND 0, 7, 14, and 21. Cells isolated at PND 0 and 7 were nearly all 3βHSD-negative, consistent with putative SLCs previously characterized as 3βHSD-negative [Ge et al, 2006]. In contrast, 90% of the cells isolated at PND 14 and 21 were faintly stained for 3βHSD, and about 5% were intensely stained for 3βHSD, perhaps implying that the putative SLCs had begun to differentiate and transition into PLCs at this stage [Ge et al, 2006]. Additionally, upon examining the differences between the faintly and intensely stained cells, it was found that the intensely stained cells were remaining fetal Leydig cells and thus were sorted and removed for all further experiments [Ge et al, 2006]. The remaining 3βHSD-negative, PDGFR-α-positive cells were then characterized to determine their potential for proliferation and differentiation. First, Ge et al. selected for LHR-

negative PDGFR-α-positive putative SLCs. These cells were found to express leukemia inhibitory factor receptor (LIFR) and c-kit, two known markers of stemness [Ge et al, 2006]. Additionally, these cells were Gata4-positive, which is a transcription factor that plays a role in Leydig cell development [Ge et al, 2006]. When plated in expansion media containing leukemia inhibitory factor (LIF) and PDGF, putative SLCs were shown to continue to expand without differentiating for over 6 months [Ge et al, 2006]. The cells remained LHR-negative and PDGFR-α-positive [Ge et al, 2006].

To assess the ability of putative SLCs to differentiate in vitro, the LHR-negative, PDGFR-α-positive cells were placed in DIM containing LH, insulin-like growth factor-I, thyroid hormone, and PDGF. After 7 days in culture, many cells became LHR-positive and began to express enzymes and proteins involved in Leydig cell differentiation and steroidogenesis [Ge et al, 2006]. These include steroidogenic acute regulatory protein (StAR), P450scc, 3βHSD, and P450c17 [Ge et al, 2006]. Additionally, these cells remained PDGFR-α-positive, and expressed Gata4 and stem cell factor-1, both of which are specific for Leydig cell development [Ge et al, 2006]. Testosterone was measured in culture media and shown to increase as a function of time in culture [Ge et al, 2006]. These experiments provided evidence that the putative SLCs were able to both proliferate and differentiate.

Lastly, the ability of the putative SLCs to repopulate EDS-treated testes and differentiate into testosterone-producing Leydig cells was assessed. LHR-negative, PDGFR-α-positive cells were isolated and fluorescently labeled. They were then injected into the testes of rats devoid of Leydig cells by virtue of prior injection of the rats with EDS. Ten days later testes were analyzed. The fluorescently labeled cells were found to repopulate the interstitial region of the

testis and to become positively stained for 3βHSD [Ge et al, 2006]. This series of experiments provided evidence for the existence of SLCs in the neonatal testis that are capable of giving rise to ALCs.

Knowing that SLCs exist in the neonatal testis and give rise to ALCs, it was then asked whether or not this population of stem cells continues to exist and play a role in the adult testis. Experiments to prove the presence and functionality of the Leydig stem cells in the adult testis involve depleting the existing population of Leydig cells and observing their regeneration. Leydig cells are specifically targeted and depleted by ethane dimethanesulfonate (EDS) [Davidoff et al, 2004; Kerr et al, 1985; Stanley et al, 2012; Teerds et al, 1999]. Within three days of EDS treatment, the Leydig cell population was depleted and serum testosterone levels were decreased [Davidoff et al, 2004; Kerr et al, 1985; Teerds et al, 1999]. Afterward, however, a new generation of Leydig cells is able to form, suggesting the possibility that the adult Leydig cells, identified by the presence of markers such as 3βHSD, P450scc and the LHR, may arise from stem cells [Davidoff et al, 2004; Kerr et al, 1985; Teerds et al, 1999]. To determine whether, in fact, the new Leydig cells arose from a stem cell population present in the adult, testicular cells that were PDGFR-α positive but P450scc-negaitve were isolated from the testes after Leydig cell depletion with EDS [Stanley et al, 2011]. PDGFR-α is a growth factor receptor that is specific to and required for the commitment of Leydig cell precursors to proliferate and differentiate [Stanley et al, 2011]. The isolated cells showed linear growth patterns when observed for 1.5 years, and they remained P450scc-negative [Stanley et al, 2011]. However, when the cells were cultured in media containing LH, they became P450scc-positive and began to produce testosterone [Stanley et al, 2011]. These data provide evidence to support the conclusion that the

precursors were indeed stem cells because they possess the ability to both self-renew and differentiate.

Within four weeks post-EDS treatment, the Leydig cell population and steroidogenic function are restored [Davidoff et al, 2004; Teerds et al, 1999]. Beginning at about day 15post EDS, 3βHSD and LHR positive cells began to appear in increasing numbers, and by day 56, the Leydig cell population was almost completely restored to its original size of about 25 million cells [Stanley et al, 2011; Teerds et al, 1999].

Seminiferous tubules were separated from the interstitium four days after rats were EDS treated [Stanley et al, 2011]. There were no 3βHSD -positive cells assocated with the tubules or the interstitium, which were cultured separately [Stanley et al, 2011]. When tubules were cultured in media containing LH, 3βHSD -positive cells appeared on the surface of the tubules, and the cells expressed StAR and P450scc [Stanley et al, 2011]. This cell population was not present in the cultured isolated interstitium [Stanley et al, 2011]. It was also found that after two weeks of culturing tubules with media containing LH, testosterone was detected in the media, indicating the cells had differentiated. Overall this confirmed that Leydig cells formed from precursor cells associated with the seminiferous tubules [Stanley et al, 2011].

To confirm that these precursors were stem cells, the capability of the cells to proliferate and differentiate was examined. First, cells on the surface of the isolated seminiferous tubules were EdU-labeled during the first 72h of culture [Stanley et al, 2011]. Then, after four weeks, the cells were examined. A population of the cells remained EdU-positive only, while some EdU-positive also became 3βHSD positive, indicating their entry into the Leydig cell lineage [Stanley et al, 2011]. These are the qualities expected of stem cells. To further examine whether the cells

on the tubule surfaces were indeed stem cells, tubules were cultured for 7 weeks in media containing LH [Stanley et al, 2011]. Then, the tubules were stripped of their newly formed Leydig cells with EDS, and cultured again in media containing LH. It was reasoned that if the cells that gave rise to Leydig cells in fact were stem cells, new Leydig cells would again reappear on the tubule surfaces [Stanley et al, 2011]. Indeed, cells positive for 3βHSD and P450scc did reappear and testosterone production was consistent with the appearance or reappearance of these cells [Stanley et al, 2011]. These results confirmed the presence of the stem cell population and the ability of these cells to both proliferate and differentiate. The ability of the putative stem cells to successfully proliferate and differentiate without the blood vessels and immune cells of the testis niche suggests that those components of the testis may not be necessary for the successful development of Leydig cells.

These studies suggested that the seminiferous tubules might represent the niche for SLCs. With this in mind, the ability of growth factors and cytokines deriving from the seminiferous tubules that were previously identified via microarray analysis to influence the proliferation and/or differentiation of the SLCs was assessed using the tubule culture system [Li et al, 2016]. The observed temporal separation observed between proliferation and differentiation in this system proved to be advantageous in discerning which factors had an effect on one phase or another. To assess effects on proliferation, tubules were incubated with LH plus a given factor for the first week, then only LH for weeks 2 and 3. To examine effects on the differentiation of the SLCs, the tubules were cultured with LH only for week 1, then LH plus a specific factor for weeks 2 and 3 in culture. The factors that positively influenced proliferation include PDGFAA, PDGFBB, activin, FGF2, and SAG (an agonist of DHH) [Li et al, 2016]. These effects were

demonstrated by measuring both EdU labeling and testosterone production. One of the most important findings of this series of experiments was that DHH was concluded to have the ability to commit SLCs on the path to differentiating into ALCs [Li et al, 2016].

Then, Li et al set out to determine a possible marker for SLCs. CD90 was determined to be a possible marker for locating and identifying SLCs on the surface of the tubules [Li et al, 2016]. CD90 was chosen in part because its message levels were found to be upregulated in SLCs, then downregulated/off in the PLC stage in previously analyzed microarray data [Li et al, 2016; Stanley et al, 2011]. CD90 was shown to localize to cells on the surface of freshly isolated tubules, which has been shown to be where the stem cells reside [Li et al, 2016]. Myoid cells, which also reside on the tubule surface and stain for desmin, did not stain positively for CD90, further confirming that distinct populations exist and that CD90 may be specific for the SLC population [Li et al, 2016]. Cells on the surfaces of the tubules were isolated from the tubules via collagenase digestion and the heterogeneous cell population was then stained for CD90. The stained isolated cells were then sorted and cultured. CD90+ cells were able to produce testosterone when cultured with LH+SAG (the DHH agonist) [Li et al, 2016]. CD90- cells in the same culture conditions produced no testosterone [Li et al, 2016]. This provided the evidence that CD90 could be used as a possible marker for stem Leydig cells. The use of these cells enables factors involved in stem cell function and dysfunction to be identified apart from the seminiferous tubules themselves.

Fetal onset of adult disease, the role of the Leydig stem cell and the effects on Leydig cell development and steroidogenesis

When adverse effects related to Leydig cell function are observed in adulthood following an early-life exposure to a chemical or environmental toxicant, it may imply that the stem cell population was where the toxicant had its most profound effects. This is because, as previously demonstrated, it is this stem Leydig cell population rather than the adult Leydig cell population that is present during the time of the exposure. Indeed in utero exposure to phthalates have been shown to cause an increase in testosterone production either through causing an increase in Leydig cell number or by stimulating testosterone production directly when given 1 and 10 mg/kg/day of DEHP [Ge et al, 2007; Zhang et al, 2008]. This observed increase in testosterone production is sufficient to advance the timing of puberty in rats [Ge et al, 2007]. However, in utero exposure to doses of DEHP of 100mg/kg bw/day and higher inhibited testosterone production. This inhibition was sustained at 6, 12 and 18 months of age after in utero exposure, which may suggest that the stem Leydig cells were affected and influence/disturb the maturation and function of the adult Leydig cells [Ge et al, 2007].

Based on the knowledge that there are SLCs present in the neonatal testis, Kilcoyne et al [2014]assessed the possible effects of in utero exposure to DBP on SLCs and the development of the Leydig cell lineage. When pregnant rats were given 500mg/kg bw/day DBP, intratesticular testosterone (ITT) levels were reduced in the male offspring. Specifically, between gestation day 17.5-21.5 (GD17.5-21.5) there was a 50-70% reduction in ITT. Additionally, there was a 40% reduction in Leydig stem cell number at GD21.5, and this deficit was maintained throughout adulthood in the rat. They also observed that, when exposed to DBP, there were changes in the expression patterns of genes necessary for steroidogenesis and for commitment to the Leydig cell lineage. Specifically, there were significant reductions in the expression of 3βHSD and StAR

[Kilcoyne et al, 2014]. Furthermore, they observed that the reduction of StAR was caused by decreased histone methylation of H3K27me3 (a transcriptional repressor). Decreased methylation of H3K27me3, resulted in increased expression of H3K27me3, ultimately causing a decrease in expression of StAR, one of its known targets. This resulted in a decrease in testosterone production. Overall, it is suggested from these results that fetal testosterone levels do impact and affect adult Leydig cell number and function, possibly by affecting the stem Leydig cell population.

Anti-androgenic effects seen from prenatal and perinatal exposures to phthalates most likely result in a reduction in testosterone produced late in gestation [Borch et al, 2006]. Normally in rats, testosterone levels peak from GD17-GD20 to ensure proper masculinization and reproductive tract development, but phthalates such as DEHP and DBP have been shown to alter testosterone produced in this window [Borch et al, 2006]. One possible cause for a decrease in testosterone is the downregulation of factors necessary for steroid hormone biosynthesis from the cholesterol precursor.

The expression levels of enzymes critical for steroid hormone biosynthesis were analyzed following in utero exposure to varying doses of phthalates—specifically DBP and DEHP. mRNA and protein expression levels were assessed for SR-B1 which is responsible for cholesterol uptake and transport into Leydig cells, StAR, TSPO, P450scc and P450c17 which are required for cholesterol translocation into the mitochondria and its subsequent metabolism, and nuclear receptors SF-1, PPARα and PPARγ [Barlow and Foster, 2003; Borch et al, 2006; Ge et al, 2007; Lehmann et al, 2004; Lin et al, 2009; Shultz et al, 2001; Thompson et al, 2004]. Statistically significant decreases in gene expression were seen beginning at 100mg/kg bw/day

around GD21 in the experiments, with the most drastic effects seen at concentration of 300mg/kg bw/day and greater when observed at GD21 [Borch et al, 2006; Ge et al, 2007; Lin et al, 2009; Shultz et al, 2001]. For example, there was a dose-dependent reduction in the gene and protein expression levels of SR-B1, StAR, TSPO, and P450scc, which is indicative of a downregulation of steroid hormone biosynthesis following in utero DEHP exposure, with the most dramatic effects seen at the highest concentrations used [Borch et al, 2006]. Additionally expression of SF-1 and PPARγ were reduced following exposure to DEHP [Borch et al, 2006]. When exposed to 500mg/kg bw/day of DBP downregulation of StAR, P450scc, P450c17, and SR-B1 was seen most dramatically at GD16, GD19, and GD21 fetal testes [Ge et al, 2007; Thompson et al, 2004].

In addition to assessing the mRNA and protein expression levels for enzymes involved in steroidogenesis, Leydig cell functional markers such as insulin-like factor 3 (Insl-3) which is necessary for testicular descent, DHH and Patched-1 (Ptc-1) which are necessary for Leydig cell differentiation, and development within the interstitial region of the testes were assessed following fetal DEHP and DBP exposures [Borch et al, 2006]. This was done in an effort to see how phthalates not only affect the steroidogenic function of the Leydig cells, but also the development of the Leydig cells themselves. When exposed to DEHP in utero, there was a dramatic decrease in the expression of Insl-3 in the fetal testis [Borch et al; Lin et al, 2009]. Additionally, following in utero 500mg/kg bw/day DBP exposure, there were increased incidences of cryptorchidism and hypospadias in male offspring, and there was increased incidence of subfertility in the male mice [Fisher et al, 2003]. Leydig cell hyperplasia was observed in fetal testis tissue at GD19 and GD21, and these areas of hyperplasia persisted postnatally in the adult male rats, showing possible prolonged effects of in utero exposure to

phthalates on male reproductive health [Fisher et al, 2003; Ge et al, 2007; Mahood et al, 2006; Shultz et al, 2001].

Serum and intratesticular testosterone levels, and spermatogenesis, also were analyzed following in utero DEHP or DBP exposure. At 300mg/kg bw/day of DEHP in utero exposure, there was a significant decrease in both serum and intratesticular testosterone production by the fetal testes compared to controls when observed at GD21 [Borch et al, 2006]. When exposed to 500mg/kg bw/day DBP, intratesticular testosterone levels were decreased 90% compared to controls at GD19 and GD21, and spermatogenesis was abnormal [Fisher et al, 2003; Shultz et al, 2001]. Additionally, serum testosterone levels in these rats were reduced at PND25, which shows the prolonged effects of in utero exposures [Fisher et al, 2003]. Anogenital distance (AGD) is a testosterone-dependent developmental process, and thus was also assessed. In utero phthalate exposure was correlated to a decrease in observed anogenital distance. At 500mg/kg bw/day, DEHP and DBP each caused a decrease in intratesticular testosterone levels. At this same dose, compared to controls, rats exposed to DEHP or DBP had a decreased AGD. [Ge et al, 2007]. AGD is most likely to be negatively affected when exposure to DBP occurs during GD15-17 [Ema et al, 2000]. At 100mg/kg bw/day of in utero DEHP exposure, there was an observed decrease in serum testosterone levels at PND21 and PND35 [Ge et al, 2007]. Reproductive tract abnormalities and decreased testosterone levels persisted in these rats into adulthood and were observed postnatally at 6, 12, and 18 months of age [Ge et al, 2007].

The above results taken together not only provide evidence for fetal effects of in utero exposure, but also suggest that there is a maintenance of impaired function of steroidogenesis in adult Leydig cells that may result from these fetal and early-life exposures.

The adrenal-testis axis and phthalates

It is imperative to recognize that while the Leydig cells primarily produce testosterone following LH stimulation, there is an additional mechanism through which Leydig cells are stimulated to produce testosterone—the adrenal gland. Additionally, while Leydig cells are a primary cell type through which phthalates have their adverse effects, they are not the only targets or means by which phthalates can cause decreased testosterone production and altered reproductive health in males. Again, the adrenal-testis axis is a target and exposure here too affects testosterone production. This observation was made when one group observed that the decrease in steroidogenic enzymes observed in fetal life from in utero exposure was not maintained throughout the adult life, but there were still long-lasting effects of the in utero exposure [Culty et al, 2008; Martinez-Arguelles et al, 2009; Martinez-Arguelles et al, 2011]

The adrenal gland facilitates Leydig cell testosterone production through production of aldosterone, which is the ligand that binds the mineralocorticoid receptor (MR) in the nucleus of Leydig cells [Ge et al, 2005; Martinez-Arugelles et al, 2009; Martinez-Arguelles et al, 2011]. A series of experiments was performed by Ge et al to determine that Leydig cells possess the MR and that it functions to produce testosterone when bound by aldosterone through a mechanism that is distinct from the canonical LH-mediated pathway of steroidogenesis in Leydig cells. mRNA for the MR was detected selectively in Leydig cells in the interstitial tissues of testes, as measured by immunohistochemistry [Ge et al, 2005]. Within the Leydig cell, staining was apparent in the cytoplasm as well as in the nucleus [Ge et al, 2005]. This data first confirmed that Leydig cells may be capable of responding to mineralocorticoids such as aldosterone through the MR that they possess.

To assess whether there were binding sites in Leydig cells for aldosterone, sections of Leydig cells were incubated with [^{3}H]aldosterone. Binding was analyzed by scintillation counting which measures the amount of radioactive material bound—in this case, the amount of aldosterone bound to Leydig cells after incubation. This confirmed that the Leydig cells possessed [^{3}H]aldosterone binding sites, which further confirmed the presence of the MR in Leydig cells [Ge et al, 2005]. To then determine the effects of MR stimulation on Leydig cells, Leydig cells were incubated with increasing concentrations of aldosterone and testosterone production was measured [Ge et al, 2005]. Testosterone production was found to increase as a function of incubation with increasing aldosterone concentrations [Ge et al, 2005]. To prove that this was a direct result of MR activation, Leydig cells were incubated with both aldosterone and an MR antagonist. When this was done testosterone production was greatly hindered [Ge et al, 2005]. Finally, to demonstrate that the MR stimulation of testosterone production acts independently of LH stimulation of testosterone production in Leydig cells, Leydig cells were incubated with both aldosterone and LH, and testosterone production was measured. When testosterone production was measured and compared to testosterone produced by Leydig cells incubated with aldosterone alone, there was an increase in testosterone produced by those cells incubated with both ligands, suggesting that the two hormones act in two distinct pathways [Ge et al, 2005].

Knowing the mechanisms through which the adrenal gland is able to stimulate testosterone production in the Leydig cell, the possibility for phthalate exposure to disrupt this process was analyzed. First, the effects of phthalate exposure on the nuclear mineralocorticoid receptor, which stimulates the Leydig cell to produce testosterone upon ligand binding, were

determined [Martinez-Arguelles et al, 2009;]. Studies found that in utero DEHP exposure caused decreased MR expression in adult rat Leydig cells. These in utero effects were observed at PND60 in adult rat Leydig cells [Martinez-Arguelles et al, 2009]. There were both decreases in the mRNA and protein expression levels of the MR in the PND60 testis at doses starting at 100mg/kg/day [Martinez-Arguelles et al, 2009]. Furthermore, downstream genes regulated by the MR were affected in the testis by in utero DEHP exposure [Martinez-Arguelles et al, 2009]. The deregulation of the MR by DEHP occurred through changes in methylation of the MR-promoter [Martinez-Arguelles et al, 2009].

Additionally, effects of phthalate exposure on the ability of the adrenal gland to produce aldosterone were assessed [Ge et al, 2005; Martinez-Arguelles et al, 2011] based on the hypothesis that a decrease in aldosterone production by the adrenal gland could lead to a decrease in testosterone produced by the Leydig cells because the MR is not properly activated. It was observed that not only does DEHP cause decreased expression of the MR in Leydig cells, but it also inhibits the synthesis of aldosterone by the adrenal gland [Martinez-Arguelles et al, 2011]. These two inhibitory actions together cause a reduction of Leydig cell steroidogenesis, and may provide insights into the mechanism by which in utero exposures can have sustained effects throughout adulthood in males.

The above literature review provides evidence for the negative effects of phthalate exposure on testosterone production and reproductive health and development in males. However, through in vivo experiments, the effect of exposures on the Leydig cell directly is extremely difficult to assess. The metabolism of phthalates in the body must be taken into account. Additionally, as described above, the adrenal-testis interactions are of extreme

importance. While the experiments by Martinez-Arguelles et al advocate on behalf of alterations in testosterone production distinct from changes in steroidogenic enzyme capacity, there is still a change in Leydig cell development evidenced by altered MR expression on the Leydig cell as a result of exposure to phthalates. Thus, it is critical to be able to examine the effects of phthalates on the development and function of the Leydig cell, beginning with the stem Leydig cell population. The seminiferous tubule culture system begins to tackle this by removing the testis from the whole body system and allows for the progression of the stem Leydig cell in its niche to the PLC, ILC and ALC phases to be assessed. While this allows for the development of Leydig cells from their stem cell population to be assessed in the context of the niche, this still does not allow for direct observation of the effects of phthalate exposure on the Leydig cell alone to be determined. In order to determine what the effects of exposing Leydig cells directly to phthalates may be, the stem Leydig cells must be digested off of the surface of the tubules and exposed only to the niche factors known to be necessary to facilitate their proliferation and differentiation to determine how phthalate exposures may alter one or the other in the Leydig cell. Once these mechanisms are known then it becomes possible to add back the layers involved when analyzing the effects of exposures such as the tubules, the adrenal-testis interactions, and the whole body.

INTRODUCTION

Leydig cells reside the interstitial compartment of the testis and are the primary source of testosterone (T) produced in males [Chen et al, 2009; Habert et al, 2011]. Adult Leydig cells (ALC) develop during puberty from a stem Leydig cell (SLC) population that is present in the neonatal testis and localize to both the peritublar and perivascular regions of the interstitial space

of the postnatal testis [Zhang et. al, 2008]. Once they are established in the testis, the ALCs make T and rarely turnover throughout the adult life of the male [Benton et al, 1995; Teerds et al, 1999]. ALCs form in three phases from the SLCs that are present in the testis—progenitor, immature, and then finally adult Leydig cells [Ge et al, 2006; Zhang et al, 2008]. ALCs are positive for 3β hydroxysteroid dehydrogenase (3βHSD), an enzyme that is crucial in the production of T and is specific for steroidogenic cells [Zhang et al, 2008].

It has been demonstrated that ALCs can be eliminated from the testis by treatment with the alkylating agent ethane dimethanesolfonate (EDS), which selectively targets and kills the ALCs [Davidoff et al, 2004; Kerr et al, 1985; Stanley et al, 2012; Teerds et al, 1999]. Following their depletion functional, T-producing ALCs are restored to the testes [Davidoff et al, 2004; Kerr et al, 1985; Teerds et al, 1999]. It has been demonstrated that the ALCs arise from SLCs that remain present in the testis throughout the life of the male [Stanley et al, 2011]. It has further been determined that SLCs reside on the surface of the seminiferous tubules [Li et al, 2016; Stanley et al, 2011]. There may also be stem cells in the perivascular space within the testis [Stanley et al, 2011; Zhang et al, 2008]. Culture of the tubules isolated from EDS-treated rats results in temporal separation between the proliferation and differentiation of the SLCs associated with the tubules [Li et al, 2016; Odeh et al, 2014]. Proliferation of SLCs is high the first week in culture, declines by week 2 and thereafter, and differentiation of the progeny occurs beginning week 2 [Li et al, 2016; Odeh et al, 2014]. This is consistent with what is observed in vivo [Li et al, 2016; Teerds and de Rooij et al, 1989; Teerds and Rijntjes et al, 1989]. It seems likely that both phases, proliferation and then differentiation, are regulated by niche factors released from the seminiferous tubules [Li et al, 2016]. Additionally, it has been shown that

SLCs can be isolated amongst a heterogeneous cell population from the seminiferous tubule and interstitial tissue surfaces and cultured in vitro [Li et al, 2016]. These cells, as is the case of those associated with the tubules, are able to proliferate and then differentiate in vitro, when they are given the appropriate factors that stimulate them to do so [Li et al, 2016].

Endocrine disruptors are abundant in the environment and pose a threat to humans on a day-to-day basis. These compounds can occur naturally in the environment, or as a consequence of man-made chemicals/compounds [Gregoraszczuck and Kovacevic 2013; Kopras et al, 2014]. Chemicals and toxicants are classified as endocrine disruptors based on their ability to affect the mechanisms through which endogenous hormones regulate cellular functions and thus to affect the biosynthesis and/or functioning of steroid hormones [Akingbemi et al, 2001]. There are windows of sensitivity during which endocrine disruptors can have dramatic effects on growth and development because of the precise developmental regulation the body is under as it matures [Zhang et al, 2008].

There are multiple routes through which humans can be exposed to endocrine disrupting compounds. Most common routes of exposure include ingestion, dermal contact and inhalation [Martinez-Arguelles et al, 2014]. If a mother is exposed, this exposure can affect the fetus through metabolites in umbilical cord blood and amniotic fluid [Martinez-Arguelles et al, 2014; Silva et al, 2004]. This exposure can continue postnatally through transmission through breast milk [Main et al, 2006; Martinez-Arguelles et al, 2014]. Often, it is the metabolites of the chemicals that are toxic and are transmitted from mother to fetus after the mother has been exposed [Silva et al, 2004]. These modes of primary and maternal-to-child exposure are shared among many endocrine-disrupting compounds. Additionally, there is the possibility for

occupational exposure to endocrine disruptors associated with companies manufacturing medical devices, plastic toys, and/or plastic containers that contain phthalates or other endocrine disruptors [Buckley et al, 2012; Kavlock et al, 2006; Kilcoyne et al, 2014].

The objectives in the present studies were: 1) to isolate SLCs from the surface of the tubules as well as the interstitial tissues; 2) to determine whether, as with the cells associated with the tubules, the isolated cells would proliferate and differentiate and whether the temporal separation between the two processes could be maintained; 3) to determine the regulatory molecules involved in SLC proliferation and differentiation; and 4) to assess the effects of phthalates on the development of functional Leydig cells from stem cells.

MATERIALS AND METHODS

Chemicals

M-199 medium and Click-iT EdU (5-ethynyl-2'-deoxyuridine) kit were from Invitrogen (Carlsbad, CA). Collagenase, and Platelet-derived growth factor BB (PDGF-BB) were from Sigma Aldrich Company (St. Louis, Missouri). Fibroblast growth factor-2 (FGF2) was from Peprote Clinick (Rocky Hill, NJ). Bovine LH (USDA-bLH-B-6) was provided by the U.S. Department of Agriculture Animal Hormone Program (Beltsville, MD). Insulin/Transferrin/Selenium (ITS) were from Corning (Manassas, VA). DMEN/F12 Medium was from Gibco by Life Technologies (Grand Island, NY). Smoothened agonist (SAG), a Desert Hedgehog (DHH) agonist, was from Cayman Chemical Company (Ann Arbor, Michigan). Mono-(2-ethylhexyl) phthalate (MEHP) (P1073) was purchased from Tokyo Kasei Kogyo Co., LTD (Japan). Traizol reagent for RNA extraction is from Ambion by Life Technologies

(Carlsbad, CA). Reverse Transcription Master Mix for cDNA synthesis is from Applied Biosystems (Foster City, CA). 2x Taqman qPCR master solution is from Thermoficher (Halethorp, MD).

Animal Studies

Young adult (~3months of age) male Brown Norway rats were obtained through the National Institute on Aging, supplied by Harlan Sprague Dawley Inc. (Indianapolis, IN). Animals were housed in Johns Hopkins School of Public Health animal facilities, in controlled light (14 hours in light, 10 hours in dark) and temperature (22^0C) with free access to water and rat chow. All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals according to protocols approved by the Johns Hopkins Animal Care and Use Committee.

Isolation and culture of seminiferous tubules from adult testis

Rats were given a single intraperitoneal injection of EDS (75 mg/kg body weight) to eliminate the existing adult Leydig cells from the testes. Rats were killed by CO2 four days after EDS, at which time the adult Leydig cell population was eliminated. Testes were decapsulated and the seminiferous tubules and interstitial tissues were mechanically separated with fine forceps under a dissection microscope. Tubules were placed in DMEM/F12 and M-199 medium supplemented with 0.1% BSA, 15mm HEPES, sodium bicarbonate (2.2μg/ml), penicillin/streptomycin (100U/ml and 100ug/ml), and insulin/transferring/selenium (10ug/ml;

5.5μg/ml; 5ng/ml). Tubules were cultured in 10ng/ml LH for up to 28 days. Culture media was collected and frozen for subsequent RIA testosterone assays.

3Beta-Hydroxysteroid Dehydrogenase Activity

3βHSD enzyme activity was examined in both seminiferous tubules and isolated SLCs. To examine activity in tubules the tubules were washed with PBS and dried at room temperature for 20 minutes. Staining solution (0.4mM 5β-androstan-3β-ol-17-one steroid substrate, 1mg/ml nicotinamide adenine dinucleotide, and 0.2mg/ml tetranitro blue tetrazolium) was added to slides for 40 min and then removed by two successive washes in PBS. The slides were then placed in 10% formalin for 30 min and examined. Tubules were examined after 1 and 28 days in culture with LH for 3βHSD activity. To examine 3βHSD enzyme activity in isolated cells, SLCs were first washed in PBS and then dried at room temperature for 20 min. Staining solution (0.4mM 5β-androstan-3β-ol-17-one steroid substrate, 1mg/ml nicotinamide adenine dinucleotide, and 0.2mg/ml tetranitro blue tetrazolium) was added to slides for 40 min and then removed by two successive washes in PBS. The slides were then placed in 10% formalin for 30 min and examined immediately. SLCs were examined after 1 and 28 days in culture with 2μM SAG and 10ng/ml LH for 3βHSD activity.

Effects of MEHP on tubule-associated stem Leydig cell differentiation

As described above, seminiferous tubules were isolated from testes of EDS-treated rats four days after their EDS injection. Once separated and placed in culture medium, tubules were either administered 10ng/ml LH alone, 10ng/ml LH+0.2μM MEHP, or 10ng/ml LH+1.0μM

MEHP for 24 days. Treatments occurred 3-4 days apart and media was collected and frozen for RIA testosterone assay.

Isolation of peritubular and perivascular stem Leydig cells

Rats were given a single intraperitoneal injection of EDS (75 mg/kg body weight) to eliminate the existing adult Leydig cells from the testes. Rats were killed by CO2 four days after EDS, at which time the adult Leydig cell population was eliminated. Testes were decapsulated and the seminiferous tubules and interstitial tissues were mechanically separated with fine forceps under a dissection microscope. Peritubular and perivascular cells were isolated by digesting the tubules and the interstitial issues, respectively, with 1mg/ml collagenase-D in DMEM/F12 media at 34^{0}C with slow shaking. Interstitial tissues were digested first for 10 minutes, then the supernatant was collected and fresh collagenase was added and the interstitial tissues were digested for an additional 20 minutes. Seminiferous tubules were digested twice for 10 minutes each, collecting supernatant and adding fresh collagenase between the two. After the digestions, supernatants were filtered through a funnel to isolate SLCs. Following their isolation, cells were washed with PBS and then resuspended in DMEM/F12 media containing 5%FBS, 10ng/ml FGF2 and 10ng/ml PDGFBB for one week. Once 80% confluent, the cells were split into treatment groups and the media was switched to DMEM/M199/ITS-Cell free media. Cells were cultured with 10ng/ml LH alone for the first week in culture, followed by 10ng/ml LH+ 2μMSAG (an agonist of DHH) for weeks 2 and 3. Media was collected beginning after the first treatment of week 2 and each treatment subsequently thereafter and was used to measure testosterone via radioimmunoassay (RIA). Cells were treated twice a week, 3-4 days apart.

Labeling cell proliferation with Click-iT EdU

Cell divisions were monitored with the Click-iT EdU imaging kit from Invitrogen. SLCs were labeled with EdU (10μM) for 24 h and examined immediately. Labeled nuclei (red) were examined by fluorescence microscopy. Slides were coverslipped with mounting media containing DAPI, which stains cell nuclei blue. Thus, the percentage of dividing cells was determined by calculating the number of cells labeled with both DAPI and EdU out of all the cells labeled with DAPI.

Effect of phthalate exposure on SLC proliferation

The effects of phthalate exposure on SLC proliferation were assessed in two ways. To determine the effects of phthalates on testosterone production while cells were proliferating, SLCs were cultured with either 10ng/ml LH alone, 10ng/ml LH+0.01μM MEHP, 10ng/ml LH+0.1μM MEHP, or 10ng/ml LH+1.0μM MEHP for the first week in culture, followed by treatment with LH+2μM SAG for weeks two and three in culture. Media was collected after the first DHH treatment and was used measured via RIA. Treatments occurred twice a week, 3-4 days apart. The effects of phthalate exposure on cell division were assessed with Click-iT EdU. Cells were plated into an 8-chamber slide coated with polylysine and treated with either 10ng/ml LH alone, or 10ng/ml LH+.01μM MEHP and four days later EdU label was added for 24 hours and then examined immediately.

Effect of phthalate exposure on SLC differentiation

To determine the effects of phthalate exposure on differentiation, SLCs were isolated as described above, and following their expansion in medial with PDGFBB and FGF2, cells were split into four test groups. SLCs were cultured with 10ng/ml LH alone for the first week, followed by 10ng/ml LH+2μM SAG, 10ng/ml LH+2μM SAG+0.01μM MEHP, 10ng/ml LH+2μM SAG+0.1μM MEHP, or 10ng/ml LH+2μM SAG+1.0μM MEHP for week 2, and then 10ng/ml LH+2μM SAG for week 3 in culture. Media was collected after the first SAG treatment and for each subsequent treatment and was used to measure testosterone via RIA. Treatments occurred twice a week, 3-4 days apart.

qPCR

qPCR was performed to determine P450scc mRNA expression levels. RNA was extracted from SLCs from tubules that were treated with either LH alone, or LH+0.1uM MEHP during week one, as well as from SLCs from interstitial tissues treated with either LH alone or LH+0.01uM MEHP during week two. RNA was extracted by giving cells 300μL of Triazol reagent following the protocol provided by Ambion by Life Technologies. Purity of RNA in the isolated smaple was measured using the Nanodrop 2000. Following the isolation of RNA, cDNA was synthesized using the protocol and master mix provided by Applied Biosystems. Mastermix was added to the RNA samples and then the samples were run through a reverse transcription thermal cycler to synthesize cDNA. Finally, once cDNA was synthesized, the standard 96-well PCR plate was prepared in accordance with the Thermofisher protocol. Actin primers were used as a control, while P450scc primers were used to determine the expression levels of the mRNA for the enzyme in the samples being examined.

RESULTS

Seminiferous tubule-associated stem Leydig cell proliferation and differentiation

To assess the ability of the SLCs to repopulate the surface of the seminiferous tubules and differentiate into testosterone-producing Leydig cells, seminiferous tubules devoid of Leydig cells as a consequence of EDS pre-treatment were isolated from Brown-Norway rat adult testes and cultured in vitro (Fig. 2A). On the first day in culture, the tubules were 3βHSD negative (Fig. 2B) indicating a lack of steroidogenic Leydig cells. After 28 days of culture with LH however, 3βHSD+ cells appeared on the surface of the tubules (Fig. 2C). The appearance of the cells demonstrates that the seminiferous tubules provide the infrastructure necessary to allow the SLCs on their surfaces to transition from SLCs on their surfaces to differentiate into steroidogenic cells, presumacly ALCs.

Figure 3 shows that the proliferation and then differentiation of the stem Leydig cells on the surface of tubules cultured with LH occurred in distinct times of culture. Stem Leydig cell (SLC) proliferation occurred during the first week in culture with LH (Fig. 3 above), with little if any differentiation. Then, beginning at week 2 in culture, cells began to differentiate, and over the course of the following 2-3 weeks, produced increasing amounts of testosterone (Fig. 3 below). Thus there was SLC proliferation and then decline in proliferation that preceded increased cell differentiation, the latter resulting in testosterone production.

Effect of MEHP on tubule-associated stem Leydig cell differentiation

Figure 4 shows the effects of increasing doses of phthalate exposure on the differentiation of stem Leydig cells residing on the surface of the tubules. For these studies, isolated seminiferous tubules lacking ALCs were separated into three groups and cultured in vitro. The control group was given only LH. The test groups were given either LH+0.2μM MEHP or LH+1.0μM MEHP. RIA was performed to measure testosterone levels in the media. As previously described, differentiation and thus testosterone production began around week 2 in culture. Thus, for the first week in culture for all three groups, testosterone production was negligible. Beginning at week 2, there was a gradual increase in testosterone observed in the control group, with the highest amount of testosterone produced by day 24. While the trend in testosterone production was the same for the two MEHP-treated groups, the amounts of testosterone produced by the tubules exposed to MEHP were less. The tubules exposed to 0.2μM MEHP produced less testosterone than control, with the biggest gap seen at day 24. The tubules exposed to 1.0μM MEHP produced the least testosterone at each day that testosterone was measured (Fig. 4). Thus, there was a dose-dependent inhibition of testosterone produced by Leydig cells on the surface of tubules exposed to phthalates. However, whether this was a result of a direct effect of phthalate on the differentiating stem cells or an indirect effect on these cells via effects on the tubule niche could not be determined because the cells were associated with the tubules.

Isolation, proliferation and differentiation of SLCs from the surfaces of seminiferous

We wished to determine whether there were direct effects of stimulatory factors on the proliferation and differentiation of SLCs, and if phthalates affect these processes. To these ends,

seminiferous tubules from EDS-treated rats were digested with collagenase. This yielded a heterogeneous cell population that included SLCs. The cells obtained were cultured for one week in media containing growth factors known from previous studies of culture seminiferous tubules to facilitate proliferation of SLCs, namely PDGFBB and FGF2. This resulted in cells in the culture dishes reaching 80% confluence over the course of the seven days in culture (not shown). Dividing cells were labeled with EdU. Active cell division was seen during the first week of culture (Fig. 5A). After this point in time, cells were split and they were cultured in serum-free media.

To determine whether, after their proliferation, cells could be induced to differentiate, the cells were cultured with LH plus an agonist of DHH, SAG, shown to induce differentiation by cells associated with seminiferous tubules. After 7-10 days in culture, there was a gradual increase in testosterone production over time (Fig. 5B). Cells were negative for 3βHSD after 1 day in culture (Fig. 5C), whereas those cultured for 28 days with LH + SAG stained positively for 3βHSD (Fig. 5D). The staining results were consistent with testosterone production and further demonstrated that the cells had transitioned from SLCs into ALCs

Effects of MEHP on SLC proliferation

With the knowledge that proliferation and differentiation are temporally separated both in vivo and in the seminiferous tubule culture system, the possibility that MEHP may affect SLC proliferation and differentiation differently was tested. Cells were obtained from collagenase-treated tubules, and cultured in media containing the growth factors, FGF2 and PDGFBB, which have been shown to simulate proliferation of SLCs associated with the seminiferous tubules.

Then, the SLCs were split into two treatment groups—LH only or LH+0.1μM MEHP. Cells were treated twice during one week, three days apart, and after the second treatment cells were EdU labeled for 24 hours and observed the following day. Labeled cells cultured with LH alone are shown in Figure 6A. Greater numbers of labeled cells were seen when the cells were cultured with LH plus 0.1μM MEHP (Fig. 6B). These observations are quantified in the graph shown in Figure 6C; the percentage of EdU-labeled cells was greater in response to exposure to LH+MEHP than LH alone.

Next, the effects of MEHP exposure during the proliferation period on subsequent testosterone production were assessed (Fig. 7 above). For these studies, cells obtained by collagenase digestion of the seminiferous tubules were first cultured in media containing FGF2 and PDGFBB to allow for their expansion in vitro. Then, cells were split into four different test groups. For the first week in culture, cells received two treatments of either LH alone, 3-4 days apart, or of LH plus one of three concentrations of MEHP: 01μM, 0.1μM, or 1.0μM. Then, for weeks two and three, cells received only LH+DHH, again twice a week 3-4 days apart. Testosterone produced by cells cultured with LH plus the lowest dose of MEHP, 0.01μM increased compared to testosterone production in response to LH or LH plus the two higher MEHP doses (0.1μM and then 1.0μM) The observed increased in proliferative activity suggests that the increase in testosterone observed may be due to an increase in cell number, i.e. more cells producing testosterone causing an overall increase in testosterone. Preliminary qPCR results (Fig. 7 below) indicated that levels of P450scc were consistent with the testosterone results: LH+0.1μM MEHP during week one in culture followed by LH+SAG for weeks two and three resulted in increased P450scc expression compared to cells treated with LH alone.

Effects of MEHP on SLC differentiation

To determine the effects of MEHP exposure on SLC differentiation, SLCs from seminiferous tubules were isolated and cultured in media containing FGF2 and PDGFBB to allow for their expansion. Following one week in expansion media, SLCs were split into four test groups and were treated twice a week for one week, 3-4 days apart. For the first week in culture, all four groups received LH alone. For week two, cells received either LH+SAG alone, LH+SAG+0.01μM MEHP, LH+SAG+0.1μM MEHP, or LH+SAG+1.0μM MEHP. For week three, all received only LH+SAG. There was an observed decrease in testosterone produced by SLCs at each MEHP concentration compared to LH-treated controls (Fig. 8).

Additionally, in preliminary studies, interstitial tissue was isolated and cultured for a week in media containing FGF2 and PDGFBB to allow for the expansion of putative stem cells, and afterward in media containing LH+SAG, LH+SAG+0.01μM MEHP, LH+SAG+0.1μM MEHP, or LH+SAG+1.0μM MEHP. LH+SAG stimulated testosterone production. As with cells derived from the tubules, there was a decrease in testosterone produced by the interstitial tissue cells at each MEHP concentration compared to LH-treated controls (Fig. 9 above). Initial qPCR experiments to examine the expression levels of P450scc corroborated the data found from measuring testosterone. In these studies, message levels of the P450scc were determined in cells isolated from interstitial tissue treated with LH only for the first week in culture, followed by either LH+SAG or LH+SAG+0.01μM MEHP for week two, and then LH+SAG for week three. Preliminary data shows that there is a decrease in expression levels of P450scc in those cells exposed to phthalate compared to controls (Fig. 9 below). Further studies need to be performed

to determine what, if any, additional steps of testosterone production may be targeted and altered as a result of MEHP exposure.

DISCUSSION

The Barker hypothesis of the fetal origins of adult disease has laid the foundation for discerning how adverse in utero influences can cause metabolic and physiological consequences that pose health risks in the adult [De Boo et al, 2006]. This has since been expanded to study how various exposures and stressors—whether environmental, nutritional, or emotional—can alter the developmental programming that occurs in the fetus to cause disease states that persist throughout the life of an adult, and spans areas of life that move beyond metabolic health and function to include areas such as cognitive and emotional health [Roth et al, 2009]. Often, the persistent effects occur through alterations in epigenetic marks, commonly DNA methylation, that result in altered gene expression and irregular development [Roth et al, 2009]. However, not all exposures result in changes in epigenetics, and yet, there are still negative health and developmental outcomes that are seen well into adulthood following an early life exposure. In these instances, it may be plausible to wonder whether a particular stem cell population was targeted and affected by the exposures.

Leydig cells mature during puberty in rats and form from a stem Leydig cell population that is present in the neonatal testis [Ge et al, 2006; Zhang et. al, 2008]. Once they are established in the adult rat testis—at a population of about 25 million cells in the testis interstitial space—they provide the male with testosterone necessary for reproductive health and function [Benton et al, 1995; Chen et al, 2009; Zhang et al, 2008]. Additionally, unless the Leydig cells

are experimentally removed, they rarely turn over [Benton et al 1995; Teerds et al 1999]. However, if elimination of the Leydig cells does occur, stem cells in the testis are able to first proliferate to repopulate the interstitial space, and then differentiate from SLCs to ALCs and thus restore testosterone production in the male [Davidoff et al, 2004; Kerr et al, 1985; Stanley et al, 2012; Teerds et al, 1999].

Each stem cell population has a specific niche—an anatomic location with specific function that provides the architecture for the stem cells to reside [Scadden, 2006]. The niche also provides the stem cells with the appropriate signals required for their proliferation and self-renewal, or their differentiation into a specific cell lineage [Li et al, 2016; Scadden, 2006]. For the Leydig stem cell, there is recent evidence that the niche at least in part is represented by the seminiferous tubules [Li et al, 2016; Stanley et al, 2011]. The tubules provide the physical surface on which the stem cells reside, fulfilling its anatomic role [Li et al, 2016; Stanley et al, 2011]. Additionally, Sertoli cells from within the tubules secrete specific paracrine factors that serve to dictate SLC proliferation and differentiation in a precisely regulated manner [Li et al, 2016].

Among the chemicals that pose a severe threat to the developing fetus are endocrine disrupting compounds [Akingbemi et al, 2011]. Endocrine disruptors can occur naturally in the environment, or as a consequence of man-made chemicals and compounds [Gregoraszczuk and Kovacevic, 2014; Kopras, 2014]. Their general mechanism of action is characterized by delayed or otherwise altered hormone function or development [Akingbemi et al, 2011]. Due to the extreme sensitivity of many hormones in the body and the precise windows of development that are regulated in organogenesis and gestational maturation, if a hormone is synthesized too early

or in too large a quantity it can cause altered expression of a specific cellular pathway. Similarly, if hormone synthesis is inhibited or delayed as a result of an exposure to endocrine disruptors, development may be delayed or impaired [Borch et al, 2006; David et al, 2006; Huand et al, 2012].

Phthalates—synthetic diesters of phthalic acid—are a family of plasticizing chemicals used in a variety of medical devices and consumer products to provide rigidity and to also act as solvents to provide lubrication and flexibility [Buckley et al, 2012; Kablock et al, 2006; Schettler et al, 2006]. The two most commonly used phthalates are di-2-ethylhexyl phthalate (DEHP) and di-n-butyl phthalate (DBP). Phthalates have been classified as endocrine disruptors due to their ability to impair human reproductive and developmental health. Specifically, they are able to act as anti-androgenic compounds resulting in abnormal testosterone production and altered male reproductive tract development [Akingbemi et al, 2001; Zhang et al, 2008]. Additionally, the metabolites of DEHP and DBP, MEHP and MBP, respectively, have been shown to disrupt Leydig cell structure and function in males [Borchea et al, 2006; David et al, 2006; Huant et al, 2012].

Various in vivo animal experiments have looked at the effect of in utero exposure to phthalates on steroidogenic enzyme function in developing and adult Leydig cells, testosterone production and reproductive tract development in male offspring [Latini et al, 2004]. These studies have suggested that these early life phthalate exposures can have adverse effects that persist throughout the adult male life [Ge et al, 2009; Zhang et al, 2008]. Additional epidemiologic studies have found correlational evidence suggesting a link between in utero/early life exposures to phthalates (through maternal breast milk) and increased incidence of

cryptorchidism in male offspring [Krysiak-Baltyn et al, 2012; Main et al, 2006]. However, an exact mechanism for how phthalates produce their effects is unknown. Some studies have suggested that there is impairment of the steroidogenic enzymes necessary for testosterone production [Borchea et al, 2006; Ge et al, 2007; Kilcoyne et al, 2014; Lin et al, 2009; Shultz et al, 2001]. Others have suggested that is it independent of the function of these enzymes, but rather (or additionally) that there are adverse effects on the adult adrenal glands, resulting in impaired communication between the adrenal gland and the testis [Ge et al, 2005; Martinez-Arguelles et al, 2009; Martinez-Arguelles et al, 2011]. Additionally, since the adult Leydig cells are not present in the testes at the time of these early life exposures but rather stem cells that give rise to the Leydig cells, it is presumably the stem Leydig cell population that is being targeted and affected.

In order to address the questions of whether or not there are effects of phthalate exposure that directly impair SLC proliferation and differentiation, and thus adult Leydig cell function, we used both isolated seminiferous tubules and cells from the tubules, the latter including SLCs. First, we used isolated seminiferous tubules from EDS-treated rats to demonstrate that SLCs are present on their surfaces, and that over time in culture with LH the stem cells are able to proliferate and then differentiate into 3βHSD+ Leydig cells capable of producing testosterone. Sertoli cells within the seminiferous tubules communicate with the stem cells on the surface of the tubules through secretion of paracrine factors that act as cues to stimulate stem cell proliferation and then differentiation. The two phases occur separately in vivo and this temporal separation is maintained when the seminiferous tubules are cultured in vitro [Odeh et al, 2014; Teerds and de Rooij et al, 1989; Teerds and Rijntjes et al, 1989].

The seminiferous tubule culture system was then used to assess the effects of exposure to MEHP on SLC differentiation. The advantage of this system is that it can be used to examine the development of Leydig cells from stem cells apart from other organ systems in the context of their niche. The results indicated a decline in testosterone produced by SLCs associated with tubules ther were exposed to MEHP compared to controls. This suggested that phthalates may exert their actions on the stem Leydig cells. However, since the cells were still residing in their niche, the potential for the niche to be implicated in the exposure and thus have a role in causing adverse effects on Leydig cells could not be ruled out. This led to our use of a stem Leydig cell culture system, where SLCs are separated from their niche and cultured in vitro with factors that are added to the culture media, not emanating from the tubules.

Using the stem Leydig cell culture system, we were able to demonstrate that SLCs can be isolated from the surfaces of seminiferous tubules and interstitial tissues and cultured successfully in vitro. The administration of niche-specific factors was found to facilitate their proliferation and differentiation in a temporally distinct manner. For instance, when the whole tubules are cultured, it was found that PDGFBB and FGF2 are released from the niche during the first week in culture, leading to the proliferation of the stem cells on the tubule surfaces. DHH is secreted by Sertoli cells beginning the second week in culture, which stimulated SLC differentiation. In experiments, an agonist of DHH, SAG, was used. These factors can be administered in the same time frame when the isolated SLCs are cultured. We found that this approach could be used to maintain the temporal separation between proliferation and differentiation. SLCs cultured without any of these factors failed to proliferate or differentiate to

produce testosterone. Ultimately, the stem cells could be stimulated to differentiate into 3βHSD+ cells that are capable of producing testosterone.

Preliminary studies assessed the putative SLC population located in the interstitial tissue of the testis. Using the same collagenase digestion system used to isolated SLCs from seminiferous tubules, putative SLCs were isolated from interstitial tissue and cultured with LH alone for one week followed by LH+SAG, and were found to produce testosterone. While evidence from Stanley et al, 2011 rejected the existence of stem Leydig cells amongst the interstitial tissues, this may be because they were removed from the their local source of DHH secreted from Sertoli cells within the seminiferous tubules when the two tissues were cultured separately. Thus, the putative SLCs never differentiated to gain the capacity to produce testosterone. It would be worthwhile to culture the interstitium from testes pre-treated with EDS with LH+SAG to see if there are putative SLCs amongst the interstitial tissue that are capable of differentiating and producing testosterone. If testosterone is successfully produced then it may be interesting to assess the effects of factors known to facilitate proliferation such as FGF2 and PDGBFF on SLCs associated with the interstitial tissue to see if they play the same role in a more complex environment than a culture dish, that is still distinct from the niche.

The use of the isolated cells provided the ability to assess whether phthalate exposures act directly on SLCs to affect their proliferation and/or differentiation to form testosterone-producing Leydig cells. An increase in testosterone production was seen compared to controls when SLCs were exposed to MEHP while the cells were proliferating. Additionally, there was an increase in cell division in proliferating SLCs exposed to MEHP shown with EdU labeling, as well as an apparent increase in P450scc mRNA expression levels. These results suggest that the

increase in testosterone observed in these cells after phthalate exposure. The increase in testosterone produced may be a result of an increase in the number of cells capable of producing testosterone. Alternatively or additionally, there may be an increased capacity of each cell to produce testosterone. When SLCs were exposed to MEHP while they were differentiating, however, there was a decrease in testosterone produced by Leydig cells compared to controls. These results are consistent with the inhibition of testosterone produced by SLCs residing of the surface of the tubules following exposure to MEHP.

While there is an observed decrease in testosterone produced by differentiating Leydig cells, the exact mechanism through which this occurs is still to be determined. Thus, it is not known which enzymes or proteins might be responsible. However, the fact that there is altered testosterone production—namely stimulation during proliferation and inhibition during differentiation—when exposures occur directly to SLCs during their differentiation phase indicates that there is a direct effect on the developing Leydig cells, perhaps involving effects on the steroidogenic enzymes or the developing Leydig cell itself. This is supported by the preliminary results from qPCR experiments demonstrating a decrease in P450scc mRNA expression in cells exposed to MEHP while differentiating compared to controls.

It would be worthwhile to conduct additional studies of the steroidogenic enzymes, and particularly to compare results obtained to the expression of these enzymes when exposure is in the context of the niche (i.e. using seminiferous tubules rather than isolated cells). The objectives would be to determine if: 1) there are upregulated or downregulated expression levels of particular genes and proteins; and 2) whether the niche (seminiferous tubules) influences the expression of genes and proteins.

While the isolated cell culture system allowed for examining the effects of specific niche factors and environmental toxins on stem Leydig cell proliferation, differentiation and development, the population of cells that was used was heterogeneous. The cells isolated from the collagenase digestion were not solely SLCs, but included other cell types such as germ cells. During the time that these experiments were being performed, Chen et. al published a protocol for the isolation of SLCs using the CD90 cell surface marker to mark SLCs, and flow cytometry techniques to isolate and purify the SLCs. Thus, it is now possible to sort the heterogeneous cell population that is initially retrieved after collagenase digestion to end up with a homogenous CD90+ population of stem Leydig cells. It would be worthwhile to repeat the experiments performed above using the CD90 sorting technique to completely isolate the SLC population. This way, the possibility for any cell-cell communications or interactions even outside of the niche are eliminated and the effects of niche-specific factors and environmental toxins on the stem Leydig cells directly can be examined.

In addition to eventually placing these studies back in the context of the testis and whole body environments, it should be noted that the data reported is from exposures of cells to only MEHP. However, in reality, these sorts of isolated exposures are rare. Instead, humans and animals are exposed to combinations of phthalates such as DEHP and DBP, or phthalates and other toxicants/chemicals that may have synergistic or additive effects. Thus, although these experiments provide data relating to the effects of and mechanisms of phthalate mediated toxicity, further studies examining the effects of multiple phthalates together should be performed in extensive detail to determine if there are indeed additive or multiplicative effects of exposure to multiple phthalates or phthalates and other chemicals. The isolated stem cell and

tubules culture systems provide a platform to perform experiments in parallel to determine effects on the Leydig cells themselves.

Overall, the above studies demonstrate the development of a novel stem Leydig cell culture system in which SLCs are isolated from the surfaces of seminiferous tubules and interstitial tissue first proliferate and then differentiate in vitro. The use of these cells allows for the identification of the effects of suspected niche-specific factors on the proliferation and differentiation of the SLCs in culture and a way to assess the effects of exposure to environmental toxicants on Leydig cell differentiation without the influences of other cells or organs.

FIGURES.

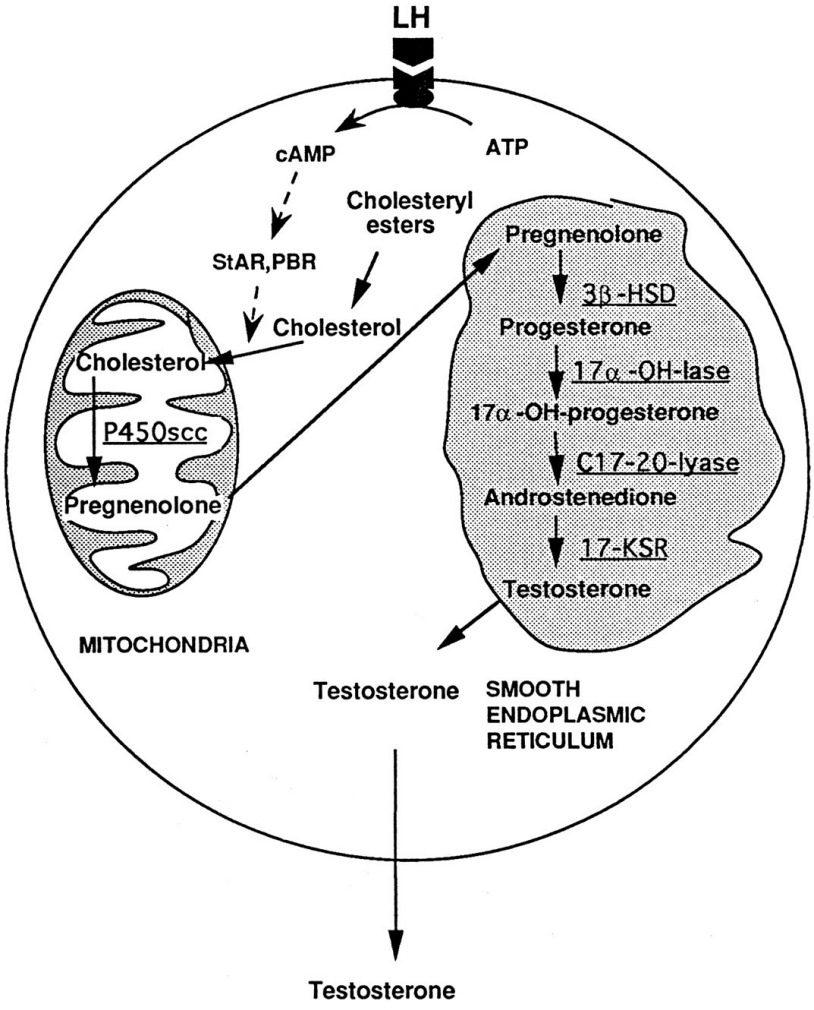

Figure 1. Steroidogenic pathway in Leydig cells. Stimulation by LH leads to increased cAMP which stimulates the transport of cholesterol into the mitochondria, where it is converted to pregnenelone by P450scc. Pregnenelone is then transported to the smooth ER where 3-βHSD converts it to progesterone. After a series of metabolic steps, progesterone is eventually converted into testosterone. *(Taken from Midzak et al., 2009)*

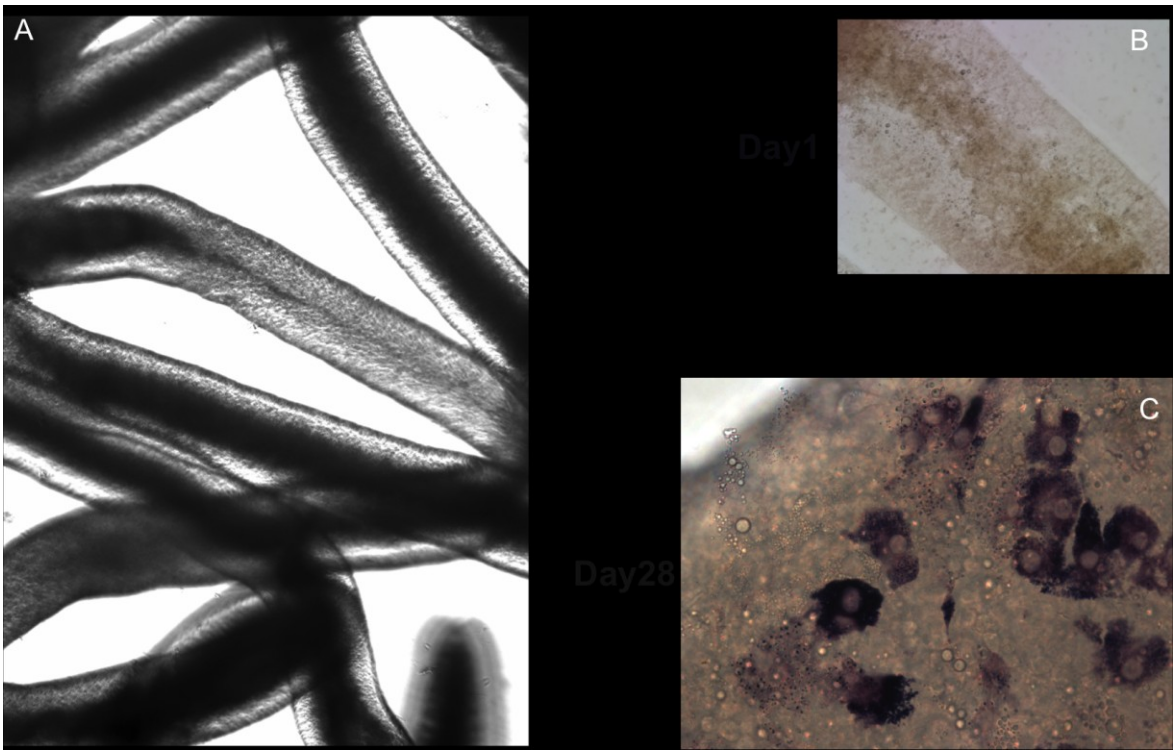

Figure 2. Leydig cell development on the surface of cultured seminiferous tubules. A: Isolated seminiferous tubules. B: There are no 3βHSD+ cells on the surface of the tubules on Day in culture. C: By 28 days in culture, 3βHSD+ cells have appeared on the surface of the tubules.

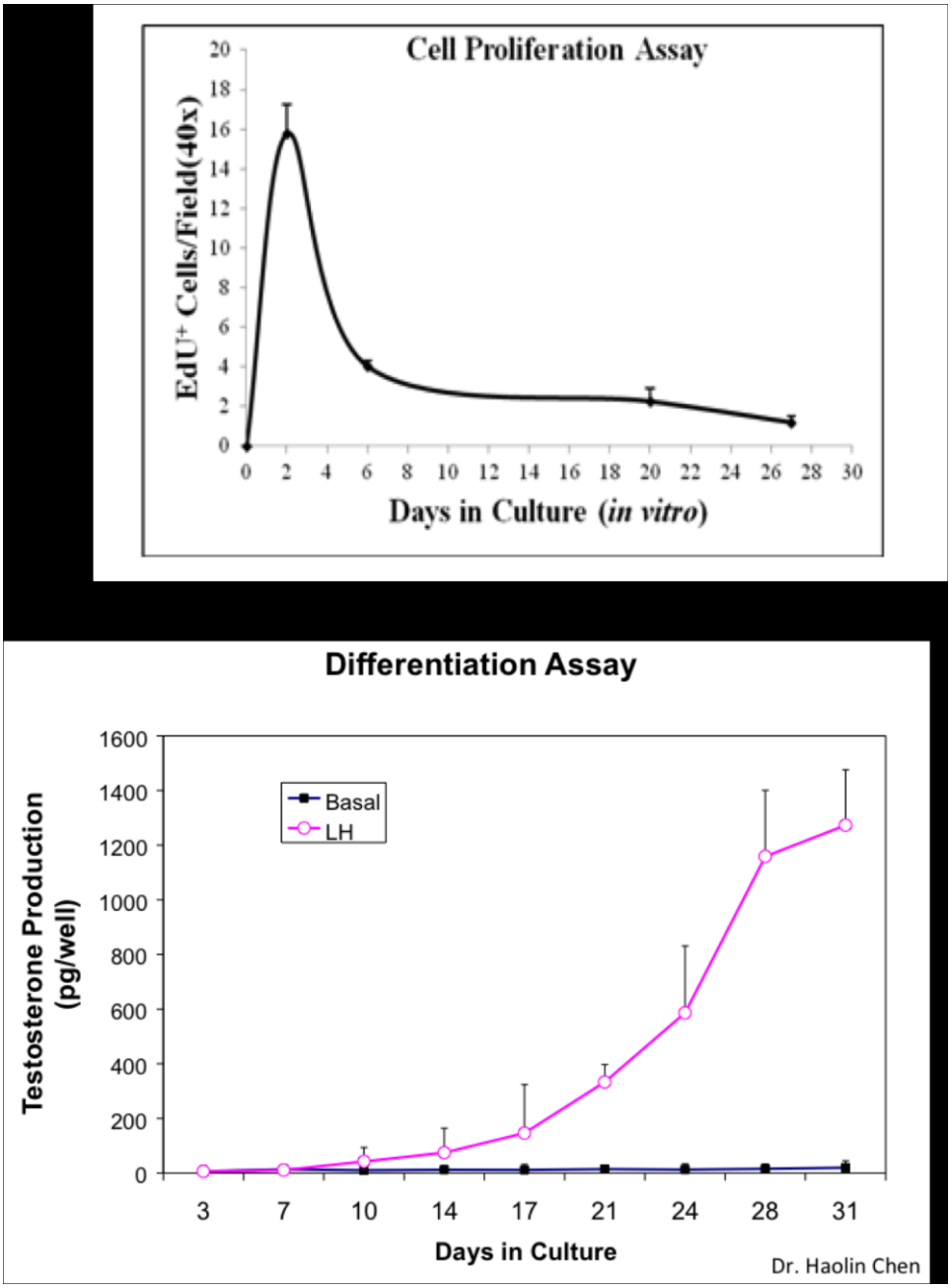

Figure 3. In vitro tubule-associated SLC proliferation and differentiation. Stem Leydig cell (SLC) proliferation (above) occurred on the surface of seminiferous tubules during the first week in culture with LH. SLCs on the surface of the tubules began to differentiate and produced increasing amounts of testosterone beginning at week 2 in culture (below). The decline in SLC proliferation coincided with the increase in testosterone produced.

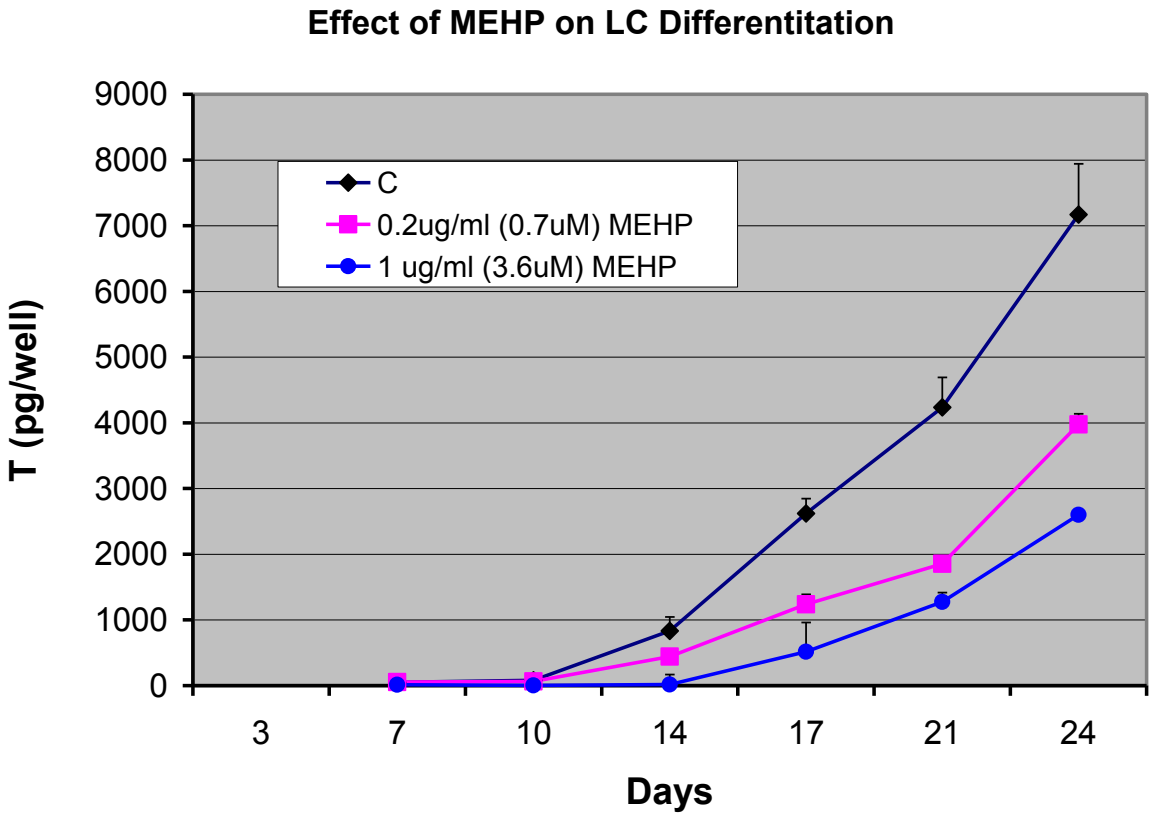

Figure 4. Seminiferous tubules cultured in vitro. Tubules were cultured with LH alone, LH+0.2μg /ml MEHP, or LH + 1μg/ml MEHP for up to 24 days. There was an observed dose-dependent decrease in testosterone produced in response to tubule exposure to MEHP.

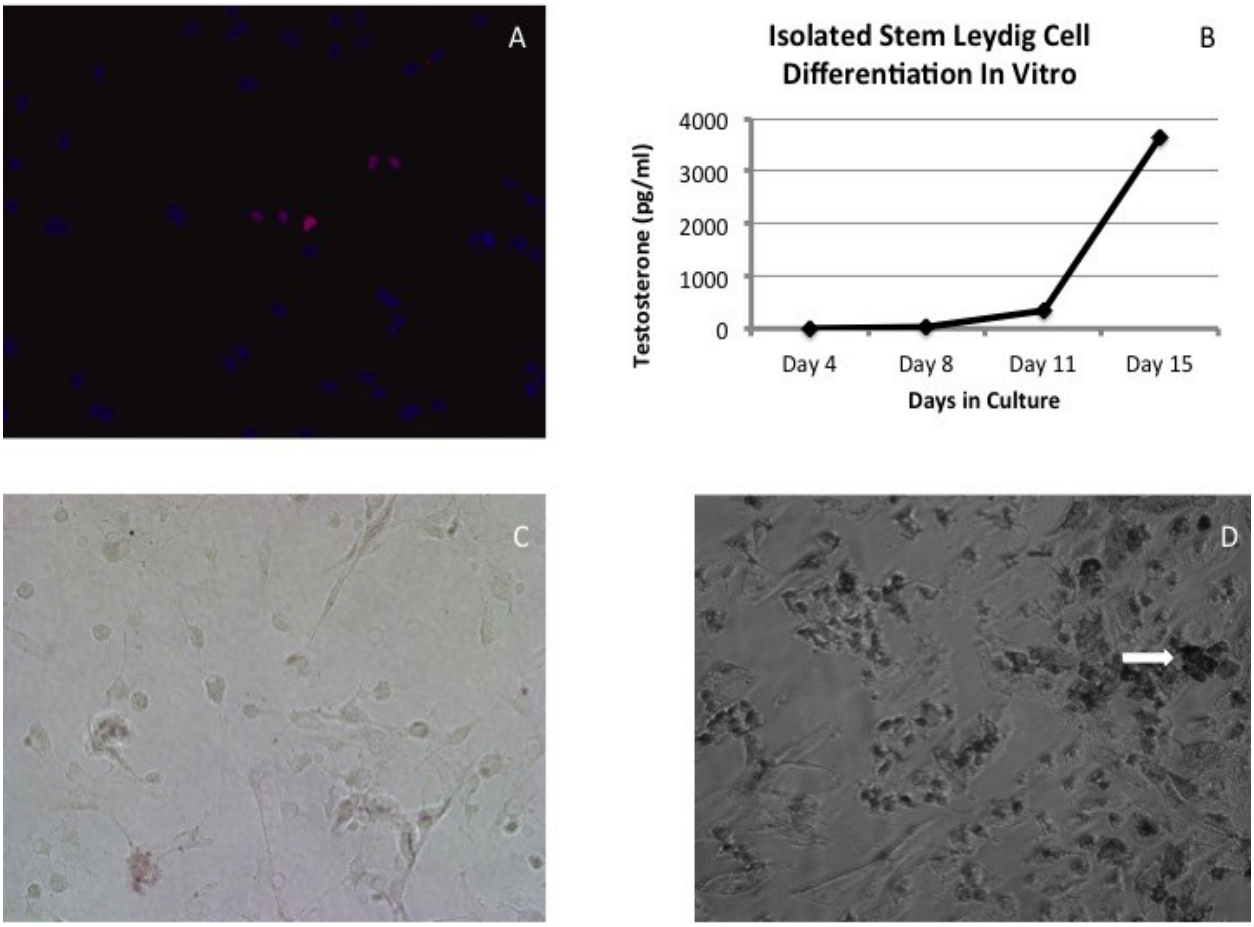

Figure 5 Proliferation of Isolated SLCs. A. EdU labeling of SLCs isolated from seminiferous tubules. All cell nuclei are stained blue with DAPI. Those that are pink are co-labeled with EdU and are actively dividing. These are presumably the SLCs. B. Time course for the differentiation of SLCs isolated from seminiferous tubules. SLCs were obtained from seminiferous tubules by collagenase digestion and cultured in vitro. Cells were cultured with LH + 2μM SAG. Differentiation was assessed by measuring testosterone in the media over time. As with isolated tubules, the cells began to produce testosterone after about a week in culture after administration of SAG. C: There are no 3βHSD+ cells amongst the SLCs isolated on day 1 in culture. D: By day 28, 3βHSD+ SLCs have appeared (arrow).

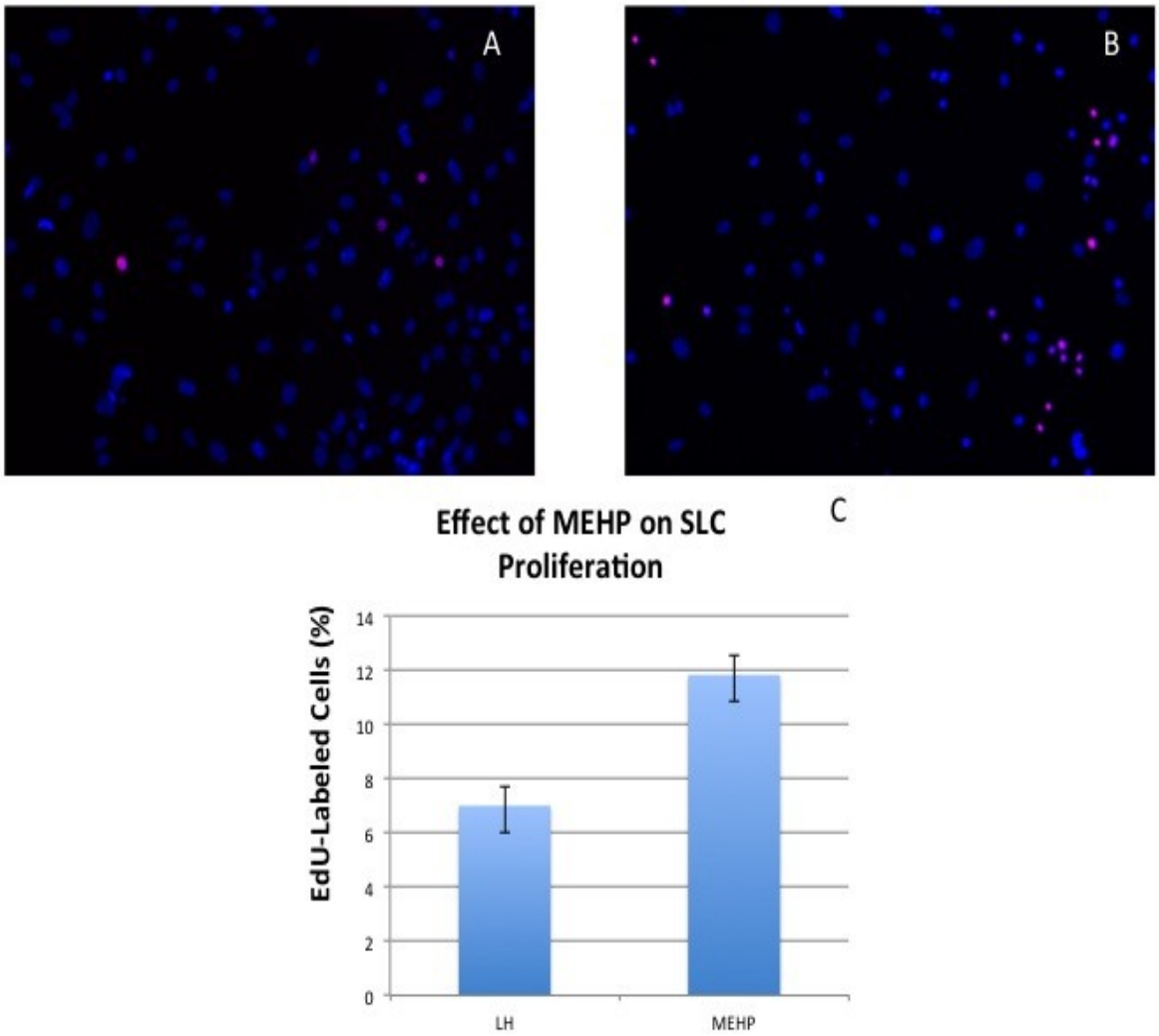

Figure 6. Proliferation of SLCs isolated from seminiferous tubules with and without MEHP exposure. Cell nuclei are stained blue with DAPI. Cells that are pink are positively labeled with EdU. A: SLCs cultured with LH alone. B: SLCs cultured with LH+0.1μM MEHP. Greater numbers of EDU-labeled cells are apparent. C: Percentage of EdU-labeled SLCs in response to LH alone or LH+MEHP in SLCs isolated from seminiferous tubules. These results indicate that exposure of the SLCs to MEHP during week 1 of culture results in increased numbers of dividing cells compared to SLCs cultured with LH alone.

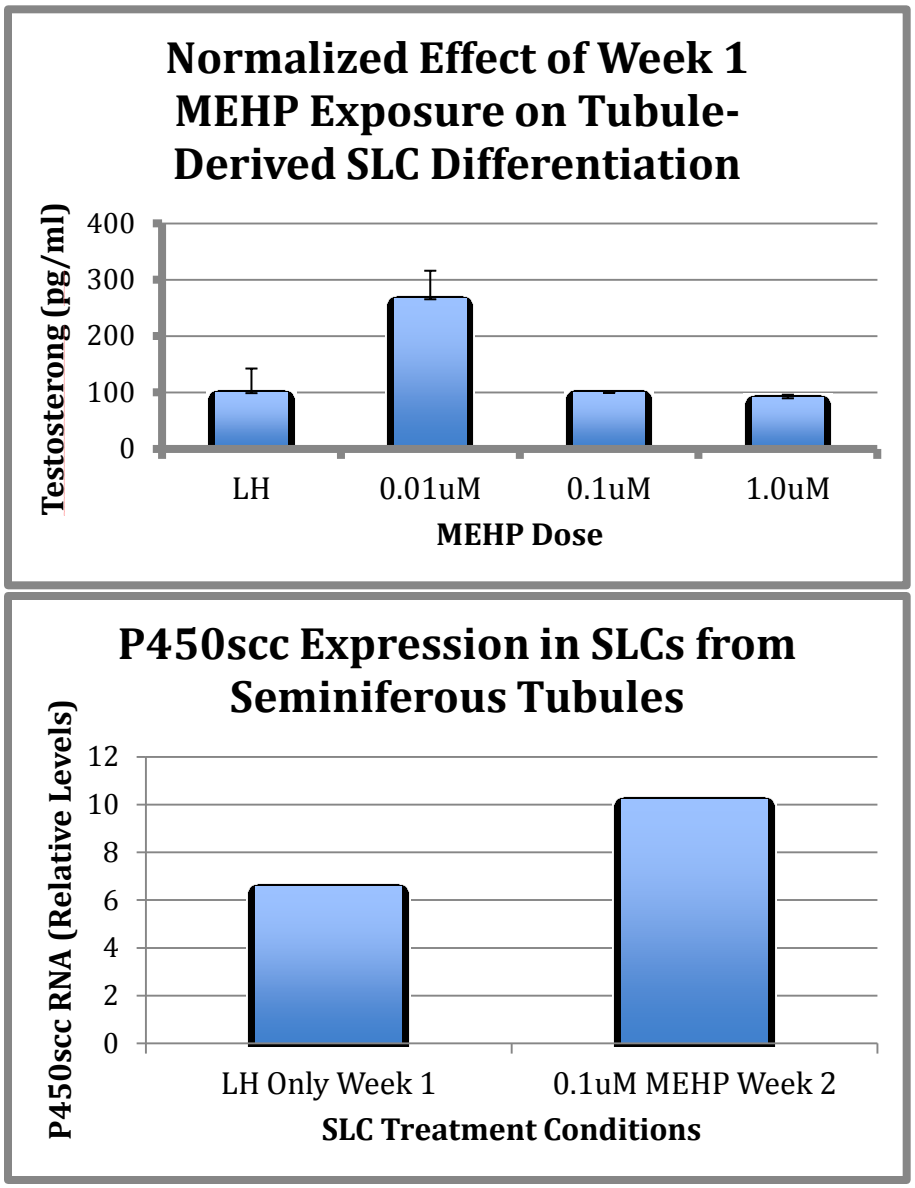

Figure 7. Above: Testosterone production by SLCs from seminiferous tubules cultured with LH alone, LH+ 0.01μM MEHP, LH+0.1μM MEHP, or LH+ 1.0μM MEHP during week 1, and then with LH+ Desert Hedgehog (DHH) during week 2 to stimulate differentiation. Increased testosterone production resulted from culture with MEHP during week 1, with 0.01μM MEHP. These results are consistent with the increased numbers of cells resulting from MEHP treatment during week 1. Below: Relative expression level of P450scc mRNA in cells treated with LH alone or LH+0.1μM MEHP during the first week in culture. P450scc expression levels are higher in those cells exposed to MEHP compared to controls. This is consistent with the results from the testosterone assay (above).

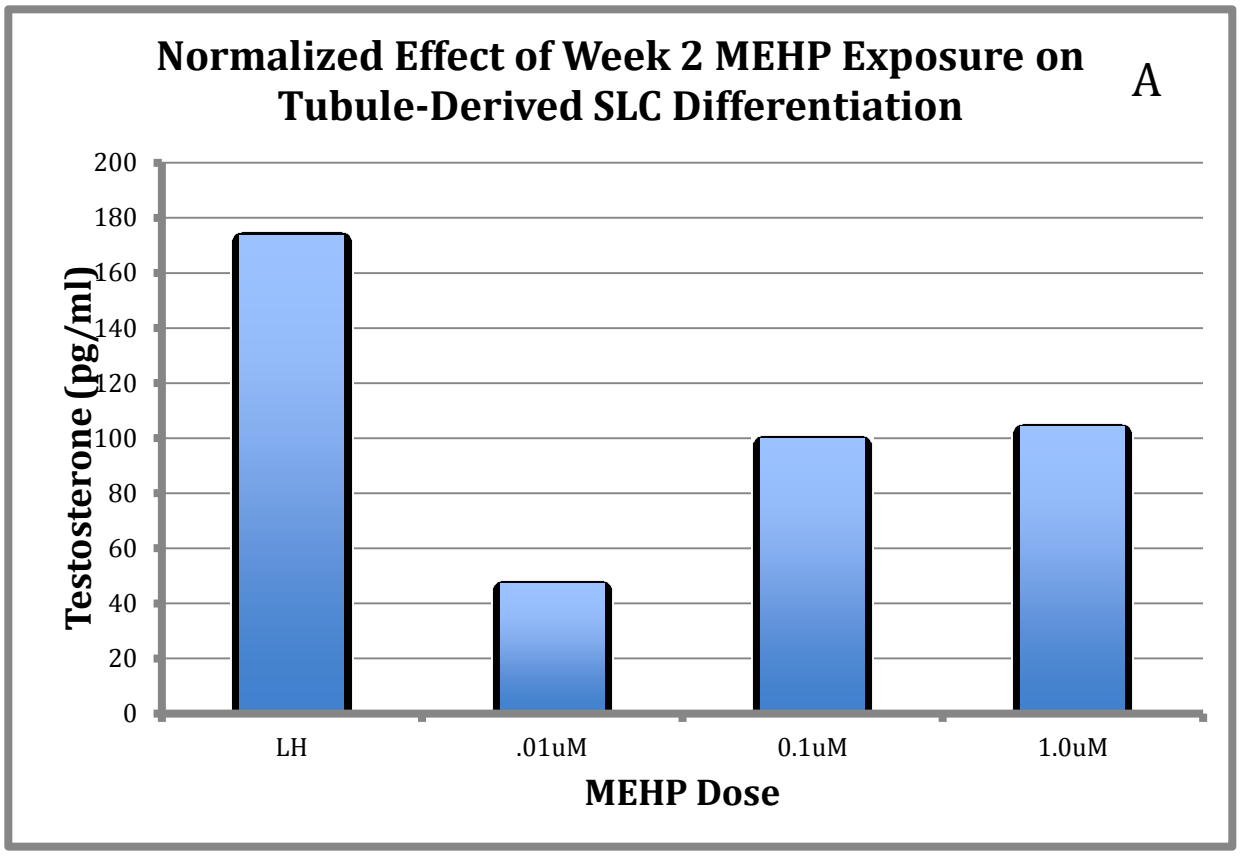

Figure 8. Testosterone produced by SLCs from seminiferous tubules in response to MEHP treatment during week 2 of culture. Testosterone production by SLCs cultured with LH alone during week 1, followed by LH+ DHH, LH+DHH+0.01μM MEHP, LH+DHH+0.1μM MEHP, or LH+DHH+1.0μM MEHP during week 2. Reduced testosterone production is seen with MEHP exposure, indicating that MEHP suppresses SLC differentiation.

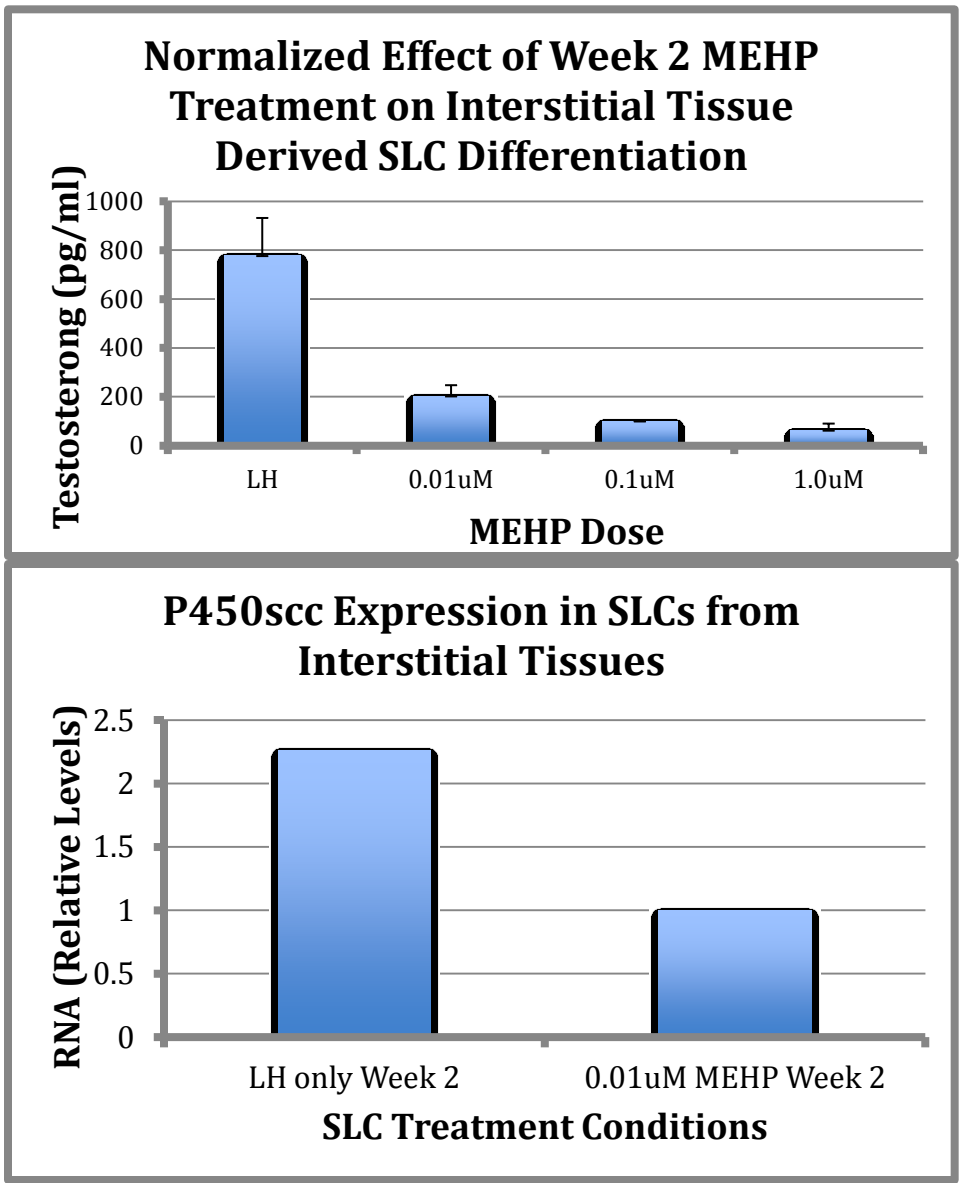

Figure 9. Evidence of putative SLCs residing in the interstitial tissues and the effects of MEHP on this population. Above: Putative SLCs isolated from interstitial tissue were treated with LH alone during week 1, followed by LH+ DHH, LH+DHH+0.01μM MEHP, LH+DHH+0.1μM MEHP, or LH+DHH+1.0μM MEHP during week 2. Similar to SLCs from seminiferous tubules, there is a decreased in testosterone production as a result of MEHP exposure. Below: Preliminary qPCR results show the relative expression levels of P450scc mRNA in cells treated with LH alone week 1 then LH+DHH or LH+DHH+0.01μM MEHP during week 2 in culture. P450scc expression levels are lower in those cells exposed to MEHP compared to controls. This is consistent with results from the testosterone assay demonstrating a decrease in testosterone in cells exposed to MEHP compared to controls.

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CURRICULUM VITAE

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EDUCATION

Johns Hopkins University Bloomberg School of Public Health Expected June 2016
Sc.M. Biochemistry and Molecular Biology *Reproductive Biology Concentration*

Johns Hopkins University May 2014
Hodson Trust Scholarship, 2010-2014 (awarded $110,000)
B.A., Biology

PROFESSIONAL WORK

Johns Hopkins Bloomberg School of Public Health Baltimore, MD
Research Position-Master of Science Student January 2015 - Present

- Worked to develop a novel stem Leydig cell culture system.
- Investigating the effects of Phthalates on Leydig stem cell regeneration, proliferation and differentiation, and testosterone production via *in vivo* and *in vitro* studies, in an attempt to discern the mechanism behind which in utero exposures can cause lifelong disease.

Johns Hopkins University Baltimore, MD
Teaching Assistant September 2015 – December 2015

- Reproductive Physiology – hold review sessions and office hours, and grade exams.

Columbia University Mailman School of Public Health New York, NY
Summer Research Position –Environmental Health Sciences Department Summer 2013

- Collaborated with Dr. Joseph Graziano and the HEALS Cohort to measure creatinine and arsenic exposure levels in study participants from Bangladesh through spectroscopy methods.
- Data was used for ongoing cancer, non-carcinogenic lung, heart and kidney disease, and nutrition studies.

New York Presbyterian/Columbia University Medical Center New York, NY
Anesthesiology Technician Summer 2011

- Assisted Pediatric Anesthesiologists by preparing IV bags, stocking equipment, and preparing operating table.
- Observed surgical procedures including heart, liver, and kidney transplants, open heart and brain surgery, and major orthopedic surgery.

Regeneron Pharmaceuticals Tarrytown, NY
Research Intern 2008 - 2010

- Investigated the function of genes by regulating MicroRNAs and developing artificial gene regulatory circuits.
- Studied cell culture and laboratory techniques
- Presented researched at local and state-wide competitions.